REMARKS

Claims 1-18 have been canceled, new claims 20-43 are pending. The new claims are consistent with the restriction requirement. Non-elected claims are cancelled without prejudice.

Support for the added claims is found as follows:

Added Claims	Support in the Specification
Claim 19	Page 2; lines 34-36; page 3 lines 14-18, 26-27, 32-36; page 6, lines 31-34
Claim 20	Page 3, lines 33-36; page 4, lines 44-46; page 5, lines 9-12; page 9 lines 42-47
Claims 21-22	Page 5, lines 9-19, former claim 6
Claim 23	Page 4 lines 44-46, former claim 4
Claims 24-25	Page 5, lines 20-28, 40-46; page 10, lines 23-37
Claim 26	Page 6, lines 4-8, former claim 10
Claims 27-28	Page 6, lines 10-11; page 13, lines 14-21; former claim 11; Example 5 at pages 19-20
Claim 29	Page 6, lines 24-29; page 13, lines 16-19, 26-30; former claim 15
Claims 30-31	Example 2 at pages 15-16; Example 5 at page 19-20
Claims 32-33, 40-43	Page 6, lines 19-22, 31-39; page 12, lines 40-41; page 13, lines 23-26; Example 5 at pages 19-20
Claims 34-35	Page 6, lines 29-39, 41-44; page 7, lines 1-4
Claims 36-39	Page 2, lines 38-44; page 3 lines 14-18, 26-27, 32-36; page 5, lines 9-12; page 6, lines 19-22, 29-39; Example 5 at pages 19-20

No new matter has been added.

Objections to the Specification

The Examiner objects to the abstract as being grammatically incorrect and an incomplete sentence. A replacement abstract is enclosed which is based on the abstract shown on the corresponding international published application. No new matter has been added.

The Examiner objects to the disclosure for containing an embedded hyperlink. The paragraph which contained an embedded hyperlink has been amended to delete the hyperlink and replaced the hyperlink with the corresponding information.

The Examiner objects to the claims because the lines are crowded too closely together. In light of the above claims amendments, the objection is believed to be rendered moot.

The Examiner objects to the title as not being descriptive. The title has been amended to adopt the Examiner's suggested title.

The Examiner noted the use of trademarks and requested that they be capitalized. The Trademarks noted by the Examiner have all been capitalized by amendment.

For these reasons, reconsideration and withdrawal of the objections to the specification are respectfully requested.

Objection to the Oath/Declaration

The Examiner objects to the oath or declaration as being defective and not complying with 37 CFR § 1.67(a) for not identifying this application by application number and filing date.

Applicants respectfully disagree. Please note that this application entered the U.S. as a National Stage Application pursuant to 35 U.S.C. § 371. A Notice of Acceptance of Application under 35 U.S.C. § 371 and 37 CFR § 1.495 was issued October 30, 2003, which stated that all requirements were met. Applicants respectfully point out that 35 U.S.C. § 371 (c)(4) and 37 CFR § 1.497 govern the oath or declaration for an application entering the U.S. National stage

from an international application. Furthermore, pursuant to MPEP § 1896, reference to the application in a Declaration under National Stage Applications (submitted under 35 U.S.C. § 371) "may refer to the international application." The Declaration of this application was filed at the same time as the National Stage Application, was signed by all inventors, and correctly references the international application by application number and filing date. The Declaration meets all the requirements as set forth under 35 U.S.C. § 371 (c)(4) and 37 CFR § 1.497, as indicated in the Notice of Acceptance. Applicants respectfully request withdrawal of the objection.

Objections to the Claims

The Examiner objected to the claims because of informalities regarding the recitation of non-elected subject matter, of the dependencies to non-elected claims, and because of improper form of multiple dependencies. In light of the cancellation of the claims and the presentation of new claims in the proper format, these objections are believed to be rendered moot. As noted, the newly-presented claims are drawn to the elected subject matter.

Rejections under 35 U.S.C. § 112, first paragraph

Former claims 1-2 and 6-16 were rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the written description requirement and for lack of an enabling disclosure. Applicants respectfully submit that the rejections based on 35 U.S.C. §112 should not apply to added claims 19-43.

Written Description Rejection

The Examiner argues that the former claims lacked written description support, on the basis that the specification does not describe a representative number of anti-HGD sequences (or HGD sequence motifs) or the structural features common to a genus of anti-HGD sequences.

Please note initially that the claims are now drawn to a method for tocopherol production in a transgenic plant and corresponding transgenic plants. There are no claims to nucleic acids or

vectors. The specification at pages 8 and 9 provides guidance for the applicability of the present method to more than a single species of antisense sequence and to more than one plant.

More specifically, Example 2 provides guidance that the exemplary *Brassica napus* HGD sequence (from which the anti-HGD is prepared) is readily derivable from sequence alignments of well known HGD sequences of *Arabidopsis thaliana*, *Homo sapiens* and *Mus musculus* (see Specification Example 2 pages 15-16). To illustrate more expressly what is disclosed in that example, Applicants enclose Exhibit 1, which is the sequence alignment of HGD sequences from *Brassica napus*, *Arabidopsis thaliana*, *Homo sapiens* and *Mus musculus* as described in Example 2. In Exhibit 1, the identical nucleotides are marked by yellow boxes.

Applicants submit that a person of ordinary skill in the relevant art (and the level of skill is high, as explained below) could use such alignments to recognize structural features common to members of the genus, as taught by the specification.

The applicable test for written description is stated in the "Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, 1, Written Description Requirements" 66 Fed. Reg. 1099, 1106 (Jan. 5, 2001). As there indicated, the written description requirement for a claimed genus can be satisfied in a number of alternative ways, such as through sufficient description of a representative number of species by actual reduction to practice, by disclosure of relevant identifying characteristics, by functional characteristics coupled with known or disclosed correlation between function and structure, or by a combination of such identifying characteristics.

Based on the high level of sequence similarity, the skilled artisan would readily understand possession of any number of sequences based on the conserved areas of the genes. For example, the skill artisan would recognize that smaller fragments of the 575-mer shown in the working example would be suitable antisense molecules. As mentioned in Example 15 of the USPTO Synopsis of Application Written Description Guidelines ("Synopsis"): "It is generally accepted in the art that oligonucleotides complementary to a messenger RNA, including fragments of the full-length complement, have antisense activity when they match accessible regions of the target mRNA." Following that rationale, Example 15 suggests adequate support

for generic claims where only a full-length antisense molecule is actually shown in the examples. It should be appreciated that the sequence of HGD from any of the crop plants encompassed by the claims could be aligned with HGD sequences the same way, to immediately envision anti-sense sequences.

In view of the species disclosed, and the common features evident from an alignment of HGD sequences from the prior art, it can be concluded that the skilled artisan would have recognized that Applicants were in possession of a genus of antisense HGD molecules useful in the claimed process.

Example 18 of the Synopsis is also relevant. That example involves a claimed invention for a method of producing a protein. There was actual reduction to practice of a single embodiment, and there was no substantial variation within the genus because there are a limited number of ways to practice the process steps. Similarly, the present specification describes a method for the production of transgenic plants which produce tocopherol by specifically inhibiting a portion of the tocopherol pathway, such as with an antisense molecule specifically described as SEQ ID NO: 1, and further provides an actual reduction to practice using a specific antisense molecule. That process is the same irrespective of the selection of the antisense molecule.

For these reasons, it is submitted that claims 19-43 are in compliance with the written description requirement.

Enablement Rejection

The Examiner asserts that the invention as claimed in the former claims was not enabled. As relevant to the added claims, the Examiner notes that the specification is enabling only for a specific sequences used in the working example. Applicants respectfully disagree for the following reasons discussed in view of the factual factors identified as relevant in *In re Wands,* 858 F.2d 731 (Fed. Cir. 1988). Applicants submit that a proper *Wands* analysis favors a conclusion of enablement.

(1) Breadth of Claims: The invention as now claimed is directed to a method of promoting the biosynthesis of tocopherol in a transgenic plant and to the corresponding transgenic plants produced therefrom. Furthermore, the claims call for inhibiting the expression of HGD in the transgenic plant. Thus, the claims are not unduly broad, but rather are focused on the suppression of one branch of the pathway metabolizing homogentisate, to direct the pathway to the biosynthesis of tocopherols (see Figure 1). This allows for the production of transgenic plants with increased tocopherol content. It is correct that the claims encompass different means of inhibiting HGD, but as explained below, the different means are disclosed in the specification and are within the ordinary skill of the art.

(2) The State of The Art/Nature of Invention/Predictability of The Art: The Examiner asserts that one of skill in the art cannot predict which unspecified anti-HGD homogentisate dioxygenase nucleic acid sequences in sense or antisense orientation or what other types of non-exemplified anti-HGD would be capable of inhibiting HGD activity when expressed in a plant because most binding sites that are vulnerable to an antisense sequence are inaccessible. The Examiner refers to *Branch et al.* (hereinafter "Branch") for support.

In response, the disclosure and teaching in Branch refer to problems with antisense molecules in the context of their therapeutic use as antisense drugs. The focus of the Branch article is on drug design and development where the non-antisense effects and binding site inaccessibility discussed were found to be impediments to rational antisense drug design (see abstract, pages 45-46). Branch discusses the antisense technology in the framework of administering the antisense molecules as a drug in the form of short oligodeoxynucleotides (ODNs). The present invention relates to the field of plant biotechnology and not to the administration of antisense molecules in the form of drugs or ODNs. Thus, it can be seen that Branch is not particularly relevant in the context of what Applicants are claiming.

As more indicative of the state of the relevant art, Applicants enclose Exhibit 2, a review article on antisense strategies in plants. J.E. Bourque, *Antisense strategies for genetic manipulations in plants*, Plant Science (1995) Vol. 105, pages 125-149 (hereinafter "Bourque"). Bourque discloses that antisense strategies for genetic manipulations in plants were well

established in the art before the priority date of the present invention. Bourque describes numerous examples of how antisense sequences have been utilized in plant systems as a method for gene inactivation and as a means of down regulating specific genes of interest. These examples encompass production of mutants, steps in metabolic pathways, identification of gene function, specificity of sequence/promoters, crop improvement, plant development to name a few (see Bourque, Table 1 at page 128, and pages 127-134). Thus, the art is actually more predictable than the Examiner has assumed.

The Examiner further alleges that absent clear and specific information in the specification regarding the identity and structure of each anti-HGD nucleic acid sequence encompassed by the claims and that each anti-HGD sequence would play a role in HGD activity, one skilled in the art would be unable to make anti-HGD sequences with predictability. In response, there has never been a requirement that every species encompassed by a claim must be disclosed or exemplified in a working example. *In re Angstadt*, 537 F.2d 498 (CCPA 1976).

The Examiner is correct to note that "anti-HGD nucleic acids" includes antisense, ribozymes and external guide sequence. The Examiner notes it also covers, citing *Waterhouse et al.* (hereinafter "Waterhouse"), the sense co-suppression of gene expression. All are dependent on a recognizable degree of homology between transgene and target sequence. In response, the specification teaches these specific strategies (pages 3-4) and provides relevant literature citations for the various strategies. The Examiner has not pointed out any reasons why the skilled artisan would be unable to practice any of these strategies. Waterhouse serves to confirm that these alternate strategies of gene inhibition using nucleic acids were known in the art.

(3) Guidance: The Examiner asserts that the application does not teach expression cassettes, vectors or plants comprising HGD nucleic acid sequences or motifs other than the antisense sequence of SEQ ID NO: 1 for generating transformed *Brassica napus* plants. The Examiner further states that given the lack of guidance, undue trial and error experimentation would be required to screen through the multitude of non-exemplified antisense and sense sequences, ribozymes or external guide sequences by producing transformation vectors and

transforming plants to identify those that when expressed in plants would be useful for inhibiting the activity of HGD. Applicants respectfully disagree.

While the working examples disclose the expression construct, vector and transformation of *Brassica napus* plants with the antisense sequence of SEQ ID NO: 1, Example 2 and the specification contain detailed guidance on how to identify suitable anti-HGD sequences, which involves aligning the HGD sequences and identifying relevant regions on which to base anti-HGD nucleic acids. That same methodology could be readily used to produce other molecules than the exemplified antisense sequence (which could be routinely screened, as explained below). Guidance is not limited to the working examples, but includes all the disclosure in the specification on how to practice the claimed invention.

Here, the specification provides detailed guidance on many different methods for identifying and making anti-HGD nucleic acid sequences, producing vectors and transforming plants therewith. For example, on page 3, lines 38-47, through page 4, lines 1-30, various methods of inhibiting HGD expression are disclosed, such as overexpression of homologous HGD sequences which lead to cosuppression, induction of specific RNA degradation by a plant with the help of an amplicon, insertion of nonsense mutations into the endogene, generating knockout mutants, or homologous recombination. At page 8, lines 34-47, through page 9, lines 1-8, additional methods for obtaining suitable artificial nucleic acid sequences with the desired characteristic are disclosed, such as coding nucleotide sequences obtained by back translation in accordance with host-plant-specific codon usage. The specification at page 12, lines 27-38, discloses various transformation vectors. Starting at page 9 line 42 through page 12 line 8, guidance for expression cassettes and appropriate promoters is disclosed. Furthermore, Example 2 teaches the isolation of a suitable anti-HGD sequence by comparison of the DNA sequences of known HGD from three species allowing oligonucleotides to be deduced (see Exhibit 1 for the sequence alignments) and Example 5 teaches the generation of transgenic *Brassica napus* plants.

The high sequence homology in HGD genes (Exhibit 1) provides guidance for identifying useful sequences in all of these embodiments.

(4) The Presence of Working Examples: The Examiner has not specifically discussed the presence of working examples and thus has not relied upon this factor in concluding that undue experimentation would be required. The Examiner has stated that the specification teaches transformation of *Brassica napus* with a vector comprising SEQ ID NO: 1, an antisense fragment isolated from *Brassica napus* cDNA library using PCR primers derived from an *Arabidopsis* HGD encoding nucleotide sequence. *See* Office Action, page 8. Applicants respectfully submit that the detailed working examples evidence the enablement of the claims. The basic steps of the method are the same regardless of the specific anti-HGD sequence used. Applicants show how to obtain an anti-HGD nucleic acid sequence, provided detailed directions on how to generate an expression cassette, vector, and transgenic plant with increased tocopherol content. *See* Examples 2 and 5. This factor clearly favors enablement.

(5) Experimentation Required: The Examiner asserts that undue trial and error experimentation would be required to screen through non-exemplified antisense and sense sequences, ribozymes or external guide sequences by producing transformation vectors and transforming plants to identify those that when expressed in plants would be useful for inhibiting the activity of HGD.

In response, Applicants agree that screening could be used to identify which anti-HGD nucleic acids would inhibit HGD in a plant cellular environment when expressed. The relevant question, therefore, is whether such screening constitutes "undue experimentation." Applicants submit that the evidence of record supports a finding that the required experimentation is not undue.

For example, as pointed out in Bourque (Exhibit 2), one skilled in the art will recognize that antisense sequences can be analyzed for their optimum length, homology to coding region, and specificity of action to optimize the desired suppressive effect. While this work may be tedious, it is routine. Exhibit 2, page 144, 2nd column.

Under the applicable law, the test for "undue experimentation" is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction

in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed. *Ex parte Jackson*, 217 USPQ 804, 807 (1982).

No evidence in the record supports a finding that the required experimentation is undue.

(6) Skill of Those in The Art: The Examiner did not address the skill of those in the art, but it is certainly very high. A person of ordinary skill in the art involved in the transformation of plants would have an advanced degree in the relevant science and several years of work experience. Such an artisan would be highly trained in the use of various plant transformation systems. Such a highly trained scientist would also have a familiarity with various regeneration systems and with various systems of gene expression in plants including antisense strategies. The very high training and experience level of those skilled in this art means that only very extensive and very unpredictable experimentation would rise to the level of "undue" in this art.

(7) Summary of *Wands* Factors: In view of the subject matter now claimed, the detailed disclosure in the specification, the inapplicability of the Branch reference, the high skill in the art and the state of the art, it is respectfully submitted that the specification fully enables one skilled in the art to practice the claimed invention without undue experimentation. Reconsideration and withdrawal of the enablement rejection is respectfully urged.

Rejections under 35 U.S.C. § 112, second paragraph

Former claims 1, 11-13 and 15 were rejected under 35 U.S.C. § 112, second paragraph, for indefiniteness. Claims 1, 11-13 and 15 have been canceled. The newly presented claims are believed to recite limitations with proper antecedent basis, do not recite a broad range and limitation together or the term "such as", and do not claim a use. In light of the cancellation of former claims 1, 11-13 and 15, the rejection is believed to be rendered moot.

Rejections under 35 U.S.C. § 101

Former claims 12 and 13 were rejected under 35 U.S.C. § 101 for recitation of a use. Claims 12 and 13 have been canceled. Therefore the rejection is believed to be rendered moot.

Rejections under 35 U.S.C. § 102(b)

Former claims 1-2, 6-7 and 10 were rejected under35 U.S.C. § 102(b) as being anticipated by *Fernandez-Canon et al.* (hereinafter "Fernandez").

This reference does not anticipate the subject matter of added claims 19-43. The reference does not teach transformation of plants or biosynthesis of tocopherol in a transgenic plant by which the expression of HGD in the transgenic plant is inhibited.

Rejections under 35 U.S.C. § 103(a)

Former claims 1-2 and 6-16 were rejected under 35 U.S.C. § 103(a) as obvious over Fernandez in view of *Tsegaye et al.* (hereinafter "Tsegaye"). According to the Examiner, Fernandez teaches a cloning/expression vector cassette (pGEX-2T) comprising an antisense HGD nucleic acid sequence (hmgA from *Aspergillus nidulans*) designated pGEX::AGMH transformed into *E. coli*. The Examiner admits that Fernandez does not teach a recombinant vector comprising a 35S or plant specific promoter and an OCS terminator functionally linked to an anti-HGD nucleic acid and a host microorganism comprising said recombinant vector; a plant transformed with said vector or microorganism comprising said vector, and a method of transforming a plant with either the vector or microorganism. The Examiner characterizes Tsegaye as teaching transformation of *Arabidopsis* with a recombinant vector construct comprising either a plant specific promoter DC3 or a 35S promoter operably linked to an antisense homogentisate dioxygenase cDNA from *Arabidopsis* that encompasses a HGD sequence motif in accordance with SEQ ID NO: 1 in antisense orientation.

The Examiner concluded that it would be obvious at the time of the invention to modify the bacterial anti-HGD expression cassette of Fernandez to substitute a 35S promoter or plant specific promoter and OCS terminator for *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis* to express an anti-HGD in a plant. The Examiner reasoned that one skilled in the art would be motivated to make this modification because it is known that an anti-HGD expression construct with the appropriate promoters are valuable for studying tocopherol production in plants and for transforming plants for greater tocopherol production as taught by

Tsegaye. The Examiner further argues that the *Agrobacterium* mediated transformation as taught by Applicants is easily applied to a wide range of plants and one would have a reasonable expectation of success of transforming *Arabidopsis* and selecting transformed plants in view of the success of Tsegaye.

In response, Applicants believe that the rejection as stated is no longer applicable. Applicants also suggest that the added claims would not have been obvious from the prior art, individually or combined.

Applicants respectfully disagree with the Examiner's characterization of Tsegaye, and submit that Tsegaye does not anticipate or obviate the subject matter of the claims.

To be anticipatory, a prior art reference must be enabling. There was no reported "success" in Tsegaye. The Tsegaye reference is an abstract which neither discloses specific constructs nor specific antisense sequences nor actual data supporting an improved tocopherol production by transgenic *Arabidopsis* plants, and does not report any results or even prove the concept of what is alleged. Tsegaye discloses a theoretical model with a prediction based on a two step process. Tsegaye does not disclose the success as characterized by the Examiner.

As explained by the court in *In re Dow Chemical Company*, 837 F.2d 469, 473 (Fed. Cir. 1988), it is insufficient to find obviousness that the prior art can be read as suggesting that an experiment would be obvious to try. There must also be an indication in the prior art that it is likely to succeed. Furthermore the court held that "both the suggestion and the expectation of success must be found in the prior art, not in applicant's disclosure." *Id.* See also, *Ex Parte Fuh*, 2002 WL 31234529 (Bd. Pat. App. & Interf. 2002) (the claim called for a treatment of certain cancers, and the prior art suggested the claimed therapy but did not determine whether the therapy would work; the Board held that: "while suggesting that growth hormone antagonists may be used in the treatment of cancers whose growth is facilitated by endogenous growth hormone, [the prior art reference] does not contain a sufficient teaching that the claimed result would be obtained. Thus, the answer fails to set forth a prima facie case that the claims are rendered obvious ... "). A copy of *Ex Parte Fuh* is attached as Exhibit 3.

The concept expounding in Tsegaye is also different from what is claimed. The reference speculates about crossing a first transgenic *Arabidopsis* comprising a construct with an *Arabidopsis* HGA-dioxygenase gene in antisense orientation, with a second transgenic plant which overexpresses HPPD. It was proposed to assess the effect on tocopherol production in different tissues of the crossed plants. There is no suggestion of increased tocopherol production in the singly transgenic plants which express the antisense sequence only.

For these reasons, reconsideration and withdrawal of the obviousness rejection is requested.

CONCLUSION

For at least the above reasons, Applicants respectfully request withdrawal of the rejections and allowance of the claims.

Accompanying this response is a petition for a three month extension of time to and including June 30, 2005 to respond to the Office Action mailed December 30, 2004 with the required fee authorization. No further fees are believed due.

If any additional fee is due, please charge our Deposit Account No. 03-2775, under Order No. 13111-00011-US from which the undersigned is authorized to draw.

Respectfully submitted,

By 

Roberte M. D. Makowski
Registration No.: 55,421
CONNOLLY BOVE LODGE & HUTZ LLP
1007 North Orange Street
P.O. Box 2207
Wilmington, Delaware 19899
(302) 658-9141
(302) 658-5614 (Fax)
Attorney for Applicants

387374

ELSEVIER

Plant Science 105 (1995) 125–149

PLANT SCIENCE

Review article

Antisense strategies for genetic manipulations in plants

June E. Bourque

Department of Biochemistry, University of Missouri, Columbia, MI, USA

Received 17 August 1994; revision received 3 November 1994; accepted 16 December 1994

Keywords: Antisense strategies; Gene expression

1. Introduction

Antisense RNAs were initially recognised in bacteria as naturally occurring mechanisms for regulation of gene expression [1] which subsequently led to the design of artificial antisense control strategies [2,3]. Even though the first engineered experiments in this area were done in mouse cells expressing complementary RNAs against thymidine kinase [4], it was in plants that the first multicellular eukaryotic organism was transformed with a foreign antisense gene [5]. The technology of antisense has been exploited to a much greater degree in prokaryotic and mammalian than in plant systems. As the literature grows with more and more successes since the first report of inhibition of the chloramphenicol acetyltransferase (CAT) marker gene in carrot protoplasts [6] and that of the chalcone synthase endogenous gene [7] in transgenic petunia plants, antisense strategies are being increasingly utilized in plant systems as a means of down regulating specific genes of interest.

The underlying mechanism(s) of gene inactivation in transgenic plants have recently been the focus of many investigations [reviews 8,9]. Previous to this, experiments involving the regulation of gene expression have been more concerned with obtaining suppression than with the molecular basis of these effects in plant systems. The phenomenon of cosuppression [10], observed in transgenic plants overexpressing an endogenous gene and thought, by some researchers [11], to involve antisense transcripts, ignited an area of research which is rapidly collecting proposed theories for plant gene silencing. In studies involving antisense [12] and sense expression of target gene sequences [10,11], the possibility of *trans* interactions has been suggested [8–10]. Transformation of plants with transgenic constructs can often increase the copy number of homologous DNA sequences, both promoter regions [13] and coding regions [14]. These transgenes, although often expressing homologous gene copies and/or promoters on separate plasmids, have been shown to contribute to epigenetic silencing [15] and to occur both in doubly transformed plants [13] and plants derived from sexual crossings [16]. Reversal of this effect has been correlated to decreases in methylation of foreign DNA [13,17] and dependence has been linked with position of repeated sequences [15] since inactivation is often reversed when the copies

0168-9452/95/$09.50
SSDI 0168-9452(94)04051-H

are segregated in subsequent progenies [18]. The effect from ectopic DNA pairing also suggests condensation of chromatin into a non-transcribable state [19]. These proposed theories on sense gene silencing all argue against the involvement of antisense RNA in *trans* inactivation [15,20]. Therefore, this review will not analyze all the factors involved in transgene silencing in plants to date, but rather discuss the specific applications of and the potential modes of action involved with plant gene regulation and inhibition via antisense strategies.

2. Rationale for antisense action

Antisense RNAs were originally defined as small diffusible transcripts [3], which lack coding capacity and bind by base pairing to specific complementary regions of a target RNA acting as a repressor of normal function and as a highly specific inhibitor of gene expression [21]. However, from the many reports on this topic, the mechanism of repressed gene expression caused by antisense mRNA remains poorly understood. The rationale behind the application of antisense genes in plants is the in vitro synthesis of complementary RNA, which subsequently hybridizes to its target RNA and prevents expression. In bacteria, the antisense RNA functions to interfere with translation most likely by annealing to the mRNA and blocking ribosome binding and initiation in the cytoplasm, but direct inhibition at the transcriptional level by binding to the extreme 5′ end of the mRNA has also been observed [3,22]. Target and antisense RNAs are generally completely complementary since they are usually transcribed from promoters in opposite directions on the same DNA segment. In natural antisense RNA regulation in prokaryotic systems, an interaction between the loop regions of the antisense RNA and the secondary structure of the sense RNA are thought to initiate reversible pairing of a few nucleotides which is followed by the formation of a stable and specific RNA duplex [3,23].

In eukaryotes, the RNA duplex [24] could potentially interfere with the normal processing of the mRNA, its transport from the nucleus or its translation in the cytoplasm [2]. It is also possible that RNA duplexes formed in the cytoplasm are very unstable, leaving no detectable signal. Complementary mRNAs have been found in the nucleus as hybrids [24] as well as in the cytoplasm [5] (Fig. 1). The reduction of the target mRNA caused by gene inactivation through antisense sequence expression has been reported to vary from several fold [5], implicating mRNA degradation, to undetectable levels [25], suggesting the inhibitory phenotype could be due to either interference of transcription in the nucleus or degradation of RNA/RNA hybrids in the cytoplasm. Details of proposed mechanisms of action of antisense RNAs in plants will be discussed later in the text.

3. Naturally occurring antisense in plants?

A few instances of naturally occurring complementary sequences in plants have been documented. In 1986, Rezaian and Symons [26], while studying the cucumber mosaic virus (CMV), noted a small single-stranded satellite (sat) RNA of 334 to 339 nucleotides. This RNA could not replicate in the absence of CMV and required CMV function for its replication. Nucleotide sequences of sat-RNA compared with the viral genome sequences displayed no extended regions of homology. However, a 5′ region of sat-RNA was found to be complementary to a small part of the minus

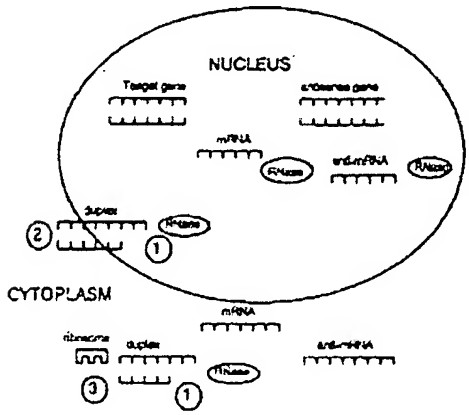

Fig. 1. Possible sites of antisense action in eukaryotes. 1, Duplex unstable and degraded; 2, blocks transport into cytoplasm; 3, prevents ribosome binding, interferes with translation.

strand sequences at the 5' end of CMV RNA 1 and 2. In vitro studies demonstrated that sat-RNA does bind to CMV RNA 3 and 4 and specifically interacts with the CMV coat protein by forming an unconventional knot-like structure. It is feasible that this binding could regulate coat protein synthesis. Natural RNAs usually contain only small stretches of complementary sequences, but Rogers [27] discovered antisense α-amylase RNAs in barley that cover essentially the full length of both type A and B α-amylase RNAs. These α-amylases are important hydrolases produced during seed germination to digest storage contents of the endosperm for use by the seedling. The complementary RNAs appear to be transcribed from a different locus and expression of both sense and antisense α-amylase transcripts are developmentally regulated.

More recently, in a study attempting to determine if upstream sequences of the rice mitochondrial *atp6* gene were involved in cytoplasmic male sterility (CMS), complementary transcripts of the *atp6* region were found, suggesting that products of transcription in the opposite direction may be involved in CMS [28]. Comparison of the complementary RNA transcription in a CMS cultivar and in an isogeneic cultivar which is fertile indicated both yielded transcripts which cover the region downstream from *atp6*, while none of these transcripts were noted in the nuclear parent of the CMS cultivar. The levels of these transcripts were higher in the non-fertile CMS line, and sometimes undetectable in the fertile cultivar. It appears that *Rf-1*, a gene product of a nuclear encoded gene which can rescue the CMS character and nullify incompatibility between a CMS-cytoplasm and a nuclear genome carrying *Rf-1*, may be responsible for the difference in transcript levels since it represents the only difference between the two lines. The complementary transcripts probably inhibit or reduce the sense transcripts and are more than likely regulated by *Rf-1*.

Analysis of transcription of the *Zea mays Bz2* locus concluded that the same genomic fragment was transcribed from both DNA strands [29]. A negative regulatory function for RNAs transcribed in the reverse orientation was not demonstrated since very low levels of the transcripts were found in tissues with repressed *Bz2* activity (low anthocyanin). However, low levels of complementary transcripts could be attributable to pairing with the sense transcripts which are then quickly degraded.

Investigations into the maize α-tubulin gene in attempts to define the spatial and temporal expression of this small family of closely related genes [30], noted a complementary transcript. Target transcripts were abundant in embryonic tissue and in dividing root tip cells while the reverse transcript was found at low levels. Conversely, endogenous transcripts were less prominent in differentiated tissue where higher levels of the complementary mRNA were present. These results suggest that the antisense α-tubulin mRNA is developmentally regulated and could possibly function to control the expression of the endogenous genes in specific tissue.

At this time, there is insufficient evidence to assign a biological function to complementary transcripts naturally occurring in plant systems. Although natural antisense mechanisms have been well documented in prokaryotes to regulate gene expression through a variety of ways [3], the role of endogenous complementary transcripts in eukaryotes has not been conclusively proven.

4. Application of antisense strategies in plant systems

As knowledge of this technology broadens, it is apparent that antisense strategies can provide powerful insights at every stage of scientific endeavor, initially to analyze promoters or transcription capabilities at the in vitro level and subsequently to ascertain tissue specificity and effect on whole plant systems at the in vivo level. In this section, examples of how antisense sequences have been utilized in plant systems as a method for gene inactivation will be presented (summarized in Table 1).

4.1. Production of mutants

Suppression of gene expression may be the only feasible method at the moment for the production of mutants in some eukaryotic systems, particularly where multigene families or multicopies of genes are present [12,31]. This mutant phenotype allows

Table 1
Examples of how antisense strategies can be applied to plant systems

Gene	Source	Host plant	Promoter	Reference
1. *Production of mutants*				
NADH-hydroxypyruvate	Cucumber	Tobacco	CaMV35S	66
Rubisco	Tobacco	Tobacco	CaMV35S	46,74
Patatin	Potato	Potato	CaMV35S	43
Nitrate reductase	Tobacco	Tobacco	CaMV35S	110
2. *Steps in metabolic pathways*				
Rubisco	*Nicotiana*	Tobacco	CaMV35S	31,38,46,53,57,74,75,7
Rubisco activase	Tobacco	Tobacco	CaMV35S	58,82a
Carbonic anhydrase	Tobacco	Tobacco	CaMV35S	72
Triose phosphate translocator	Potato	Potato	CaMV35S	41a,78
Sucrose transporter	Potato	Potato	CaMV35S	77
PFP	Potato	Potato	CaMV35S	39
Nodulin-35	Mothbean	*Vigna*	CaMV35S	55
Chalcone synthase	*Petunia*	*Petunia*/tobacco	CaMV35S/CHS	61,103b,104,105,106
3. *Identifying gene function*				
AGPase	Potato	Potato	CaMV35S	62
UGPase	Potato	Potato	CaMV35S	113
pTOM5	Tomato	Tomato	CaMV35S	6,10
PG	Tomato	Tomato	CaMV35S	89,96
pTOM13	Tomato	Tomato	CaMV35S	9,41
Flavonol synthase	Petunia	*Petunia*	MAC	45
Anionic peroxidase	Tomato	Tomato	CaMV35S	90
4. *Sequence/promoter specificity*				
H^+ ATPase A	Carrot	Carrot	CaMV35S	34
B-conglycinin promoter	Soybean	Tobacco	CaMV35S	32
BNYVV RNA	BNYVV	*C. quinoa*/	CaMV35S	17a
ras genes	Yeast/human	*Nicotiana*/*Petunia*	CaMV35S	52
5. *Crop improvement*				
PG	Tomato	Tomato	CaMV35S	14,37,87,89,95,96
ACC synthase	Tomato	Tomato	CaMV35S	65
ACC oxidase	Tomato	Tomato	CaMV35S	31b,41
PME	Tomato	Tomato	CaMV35S	103
E8	Tomato	Tomato	CaMV35S	70
PE	Tomato	Tomato	CaMV35S	40
EFE	Tomato	Tomato	CaMV35S	71
Chalcone synthase	Chrys./Gerbera	Chrys./Gerbera		19,28
Prosystemin	Tomato	Tomato	CaMV35S	59,67
B-1,3-Glucanase	Tobacco	*Nicotiana*	CaMV35S	2,64
Desaturase gene	*Brassica*	*Brassica*	napin/ACP	50
S-RNase	*Petunia*	*P.inflata*	CaMV35S	54
rolC	Agro. rhiz.	Tobacco	CaMV35S	88
6. *Plant development*				
AKR containing gene	Arab.	Arab.	CaMV35S	112
Auxin-regulated genes	Tobacco	Tobacco	CaMV35S	8
GUS		Tobacco	Chlor. a/b	13
7. *Transcript/protein relationships*				
AU-rich sequences	*zein* gene	Tobacco	CaMV35S	94
Introns	*zein* gene	Tobacco/potato	CaMV35S	11
BMV sequences				62b
Oxygen-evolving sys. proteins	Tobacco	Tobacco	CaMV35S	69
PS II 10 kDa gene	Potato	Potato	CaMV35S	98
Rubisco SS	*N. sylvestris*	Tobacco	CaMV35S	82
8. *Novel uses*				
GSA-AT	Tobacco	Tobacco	CaMV35S	44
nptII	Tn5 transposon	Tobacco	CaMV35S	111

a biological function to be determined from a specific cDNA sequence [32]. Attaining 100% inhibition of any enzyme activity may not be an absolute necessity since many null mutations might be lethal to the plant and a partial inhibition will more than likely result in a measurable change in the system [33]. Antisense RNA was used to create tobacco mutants deficient in NADH-hydroxypyruvate reductase to study the role of photorespiration in stress protection by identifying enzymes in the pathway [33]. Transformation with antisense genes also conveniently produces mutants for physiological studies, as for example with photosynthesis [34,35].

Despite being one of the major potato tuber proteins and present in all potato species thus far analyzed, no physiological role has been assigned to patatin, a 40 kDa glycoprotein. Mutants were produced by transformation of potato plants with the patatin complementary coding sequence which reduced the endogenous mRNA to less than 1% of controls and the protein by 75–90% while other members of the gene family were not as significantly reduced. No apparent changes in morphology or development of the tuber occurred bringing into question the role of patatin as a storage protein and sharply diminishing its potential for involvement in the tuberization process [36].

Although the term 'mutant' has been used by several researchers who have made alterations to genes via antisense methods, it should be noted that these alterations do not constitute a heritable change of the target gene sequence obtained through genetic means. But, since the antisense gene can inhibit closely related genes with a high degree of homology, it is assumed that the antisense trait would be dominant. However, the variability of inhibition seen in many studies may mean that antisense strategies are not reliable for the production of null mutants.

4.2. To observe steps in metabolic pathways

Antisense RNA can be a means of partially or completely repressing the synthesis of gene products in attempts to determine the effect on other proteins' contributions within the same system. To evaluate the autoregulatory function of nitrite reductase (NiR) on nitrate assimilation and N-metabolite function, an antisense approach was used where a reverse orientated NiR cDNA expressed in tobacco plants caused the impairment of NiR without modifying the nitrate reductase (NR) gene product [12]. A plant, obtained from normal growth on ammonium but chlorotic with nitrate as the source of nitrogen, accumulated 5-fold higher nitrite levels than the wild type. NR activity and mRNA were elevated under these conditions, suggesting that N-metabolites derived from nitrate reduction caused the inhibition of the NR gene independent of NR protein levels.

Extensive investigations to analyze the impact of decreased ribulose bisphosphate carboxylate (Rubisco) on photosynthesis in transgenic plants, using antisense technology, noted that the protein could be reduced to half the wild type average normally found in tobacco plants with little effect on the rate of photosynthesis, suggesting excess amounts of Rubisco exist in wild type plants [34]. Little change in phosphoribulokinase activity, electron transport [35] and chlorophyll content [35,37] were noted, suggesting no link between Rubisco and these other components of photosynthesis. A notable decline in carbonic anhydrase and the rate of CO_2 assimilation occurred but no reduction in stomatal conductance was observed, implying stomatal function is independent of Rubisco activity in the leaf [35]. When Rubisco was reduced below a threshold level, there was a concomitant decrease in plant dry weight, along with a decline in starch accumulation in the leaves and a drop in photosynthetic rate in transgenic plants [34]. These changes forced a more efficient use of the remaining photosynthate resulting in an increase of specific leaf area and light interception [34] and further reflected the interactive effect between photosynthesis and carbohydrate metabolism. Under short term conditions, the contribution of Rubisco to the control of photosynthesis was increased when irradiance levels and humidity were high and CO_2 was low [37–39].

Transgenic plants with reduced Rubisco showed an elevation in photosynthesis and an increased chlorophyll content [37,38,40] as well as a reduced chlorophyll *a/b* ratio when grown under limiting nitrogen or low light, indicating that nitrogen is reallocated to other cellular components away from

the photosynthetic apparatus [38]. Under plentiful nitrogen supply, growth reduction correlated with an increase in photosynthesis inhibition [41]. The reduction in Rubisco in transgenic tobacco plants was more than compensated for by an increase in leaf nitrate [42]. A similar decline in growth rate was noted in transgenic plants responding to a reduced carbon supply due to lower Rubisco levels as was observed in plants growing under low ambient CO_2 conditions [42]. Raising ambient CO_2 levels caused the transgenic plants to achieve growth rates comparable to wild type plants, suggesting that reducing Rubisco could improve nitrogen-use efficiency, without affecting photosynthesis and growth, if plants were grown at higher (expected future world) CO_2 levels [42].

An antisense chloroplast carbonic anhydrase gene did not appear to alter net CO_2 fixation, Rubisco activity, chlorophyll content, dry weight, or stomatal conductance [43]. Other studies on photosynthetic sensitivity assert that Rubisco activity does not rate limit photosynthesis in water-stressed plants, but rather its substrate ribulose 1,5-bisphosphate (RuBP) [44]. The reductions in Rubisco noted in the transgenic antisense plants were compensated by an enhancement in the levels of the regulatory protein, Rubisco activase [45]. Changes in Rubisco activity in vivo by carbamylation of the active site are modulated by this chloroplast enzyme. The reduction of activase levels in tobacco plants by transformation with an antisense gene [45,46] showed that Rubisco mRNA and protein levels were unaffected [45] comprising no impact on photosynthetic rates under moderate or low light intensities, however, both Rubisco and activase contributed significantly to impaired photosynthesis at higher light intensities [45,46]. Rubisco activation states correlate to activase levels, thus exhibiting a direct effect on photosynthetic rates.

In similar experiments, the introduction of the antisense chloroplast triose phosphate translocator protein gene into potato plants reduced its activity 20–30% [47] and severely decreased photosynthesis by 40–60% [48] with a noted decline in inorganic phosphate levels in the stromal compartment. A redirection of CO_2 assimilates into starch was observed as compensation for this reduction with no cost to productivity. Expression of the antisense sucrose transporter cDNA in potato plants caused a 20-fold higher concentration of soluble carbohydrates to accumulate and likewise a 5-fold increase in starch content in leaves with a dramatic reduction in tuber yield, signifying that the sucrose transporter, located at the phloem plasma membrane, is responsible for primary sugar uptake [49].

Potato plants transformed with antisense genes encoding the α- and β-subunits of pyrophosphate: fructose-6-phosphate phosphotransferase (PFP) were able to provide evidence for the role of PFP in recycling triose-phosphates to hexose-phosphates. A compensation in other phosphates resulted in no visible alterations in phenotype and only minor changes in metabolic fluxes [50].

Expression of an antisense nodulin-35 cDNA encoding nodule-specific uricase in *Vigna aconitifolia* (mothbean) was detected in transgenic root nodules resulting in lower uricase activity and a reduction in the nodule size. Peroxisome development was retarded and nitrogen deficiency symptoms appeared in the plants [51], suggesting that the availability of symbiotically reduced nitrogen in legumes is limited by a reduction in ureide biosynthesis.

The flavonoid biosynthetic pathway is an example where antisense technology has provided the basis for a model system, using floral pigmentation, for the development of studies on the manipulation of secondary metabolites in plants. Chalcone synthase (CHS) was the first endogenous gene in plants targeted by antisense RNA [7]. It was assumed that since the substrates for the CHS enzyme are colorless, any reduction in CHS enzymatic activity would result in changes in the color of flower petals [52]. This is exactly what did in fact happen, but an even more interesting observation was the occurrence of variegation in regular rings or sectored patterns on the petals which was noted in both heterologous and homologous petunia plants. Analysis of pure white flower tissue showed a complete loss of flavonoids but this could be bypassed by feeding the plant naringenin-chalcone, a product of CHS activity, confirming that the pathway beyond CHS was still functional [7]. Further investigation of this secondary meta-

bolic pathway [53] showed that steady state mRNA levels of chalcone flavanone isomerase (CHI) and dihydroflavonol (DFR), two other flavonoid specific genes, were not affected by the reduction of CHS. Where the CHS mRNA was reduced in inner corolla tissues, CHI and DFR levels remained the same. However, the CHI specific activity was 10-fold lower in white corolla tissues but remained unchanged in plants containing pale pigmented corolla tissue when compared to fully pigmented tissue, meaning that only a minute level of CHS transcript or enzyme is required for stability of the CHI protein.

ADP-glucose pyrophosphorylase (AGPase) was determined to play a role in starch biosynthesis when transgenic potato plants expressing an antisense gene containing the coding region of a AGPase subunit abolished starch formation [54]. An accompanying sharp increase in RNA levels of sucrose phosphate synthase and a reduction in major storage protein species added to the idea that storage protein genes and carbohydrate formation are linked in storage organs. Alternately, transgenic potato plants, expressing an antisense UGPase which repressed activity to 4% showed no changes in growth and development or in carbohydrate metabolism [55].

4.3. Identifying gene function

The ability to regulate the expression of specific genes in plants can be regarded as an excellent tool for assigning gene function which could have enormous commercial applications. The role of pTOM5, a ripening related gene isolated from tomato, was established in carotenoid biosynthesis [56] by expressing its antisense sequence in transgenic plants where a reduction in pTOM5 mRNA during tomato ripening correlated with yellow fruit color, pale flowers and a 97% decrease in carotenoids. The pTOM5 gene product was not implicated in carotenoid synthesis in the leaf [57]. The use of antisense RNA to inhibit polygalacturonase (PG) gene expression in ripening tomato fruit, where it is usually found in abundance, indicated that its activity is not correlated to the accumulation of the red pigment lycopene [25,58]. In studies of genes related to ethylene biosynthesis, pTOM13 was isolated from a cDNA library from clones expressed during tomato fruit ripening and in response to wounding. Subsequently, it was determined that this gene was also expressed in senescing leaves. To ascertain the function of the protein, antisense pTOM13 was used to transform tomato plants. Results showed that ethylene synthesis was strongly inhibited, suggesting the protein is involved in ethylene biosynthesis and may be part of the ACC-oxidase system involved in conversion of ACC (1-amino-cyclopropane-1-carboxylic acid) to ethylene [59,60].

Flower color is predominantly due to flavonoid, carotenoid and betalain pigments. Anthocyanins are a class of flavonoids which provide the greatest range of colors, but co-pigmented anthocyanins, with such compounds as flavones and flavonols, also produce effects on flower color. An antisense cDNA clone of flavonol synthase (FLS) transformed into petunia caused a marked decline of flavonol synthesis in flower petals and a reddening effect due to reduced co-pigmentation providing a means by which to further examine the many functions of flavonols in plants [61].

Using an antisense approach to inhibit anionic peroxidase determined that the accumulation of normally induced transcripts was prevented when the plant was subjected to wounding, exposure to ABA or fungal pathogens [62], but the level of suberin deposited in the wound-periderm was unaffected, indicating that the expression of this anionic peroxidase gene may not be essential for suberization.

4.4. Determining sequence or promoter specificity

The elucidation of specific DNA sequences in gene expression or tissue specific expression has also employed antisense methods. Constructs of the coding and 5′ non-coding region of the carrot vacuolar H^+ ATPase A subunit cDNA were expressed in carrot root tissue cells in the opposite orientation. Similar levels of inactivation were found in the bafilomycin-sensitive ATPase, ATP-dependent H^+-pumping and ATP-driven ^{14}C-*O*-methyl-glucose uptake activities in the tonoplast, but not in the Golgi, fractions. These ATPase-deficient lines, where cell expansion in young leaves and taproots was inhibited, are the first direct indications that the tonoplast H^+ ATPase

drives the osmotic uptake of water into the central vacuole to facilitate cell expansion [63].

A complementary sequence of the β-glucuronidase (GUS) gene was fused to a promoter from a β-conglycinin gene which is expressed only in seeds [64] and used to transform tobacco plants already expressing GUS. The seed-specific repression of the GUS gene noted in this study serves as a model for possible modifications in tissue specific protein accumulation.

To determine if plants can be infected by subgenomic viral RNA transcripts, sense and antisense directed cDNA clones of the beet necrotic yellow vein virus were constructed and transferred to propagation hosts. Results show only the sense constructs to be biologically active [65], signifying that expression of the sequence is necessary for viral function.

Antisense constructs of the *ras* genes isolated from yeast and mammalian cells were introduced into plant cells to study their inhibitory effect on cell growth and development [66]. Using this approach, it was determined that the *ras* sequence itself, rather than the protein or mRNA, is directly involved with the negative effect.

4.5. Crop improvement

The down regulation of polygalacturonase (PG), a key enzyme in tomato fruit ripening, probably represents the first major contribution of antisense technology to the agriculture industry in this country. PG antisense RNA expressed in transgenic tomato plants was shown to cause a reduction in PG enzyme activity as well as PG mRNA during fruit ripening [25]. It was also determined that RNA from the antisense PG gene was transcribed at a higher rate than the endogenous PG gene RNA [58]. PG expression had been implicated in fruit softening where increased levels of PG have been shown to correlate with fruit maturation [67]. Isolation of the cDNA of PG allowed for the production of antisense mutants to study its expression during ripening and gain a better understanding of its function in fruit softening [68]. Climacteric fruits, such as tomatoes, are harvested at the breaker stage [69] and are later induced to ripen with exogenous applications of ethylene. Endogenous ethylene increases autocatalytically with the onset of induction [70]. Antisense RNAs to the cDNA of rate-limiting enzymes reduced ethylene synthesis and confirmed the role of ACC synthase (1-aminocyclopropane-1-carboxylate synthase) [70], and led to the isolation of ACC-oxidase [60], both involved in the biosynthetic pathway of ethylene. Since earlier attempts to isolate ACC-oxidase activity had been unsuccessful [71], these results clearly signify the strength of antisense strategies.

Further analysis of these antisense PG mutants found PG to be involved in pectin degradation [72], while other studies showed that plants expressing antisense PG failed to solubilize pectin [73] and had elevated levels of arabinose and galactose. Similar strategies were used to ascertain that the pectin methylesterase gene is also involved in pectin metabolism but not coupled with the ripening process [74]. The E8 protein, related to ACC oxidase, was studied using antisense RNA inhibition and shown to specifically increase ethylene synthesis during ripening of detached fruit [75]. More recently, antisense RNAs directed against pectin esterase (PE), a cell wall metabolizing enzyme, indicated that PE, although not involved in tomato fruit development and ripening, plays a role in pectin de-esterification with differential isozymes expressed in various tissues [76]. When the ethylene-forming enzyme (EFE) was targeted by antisense RNAs in the tomato fruit it was determined that fruit ripening aspects were retarded in these transgenic plants and these effects became more pronounced when fruit were detached from the plant, indicating that the role of ethylene is probably more complex than previously thought and might involve a plant associated factor [77]. The use of antisense strategies to elucidate tomato fruit ripening [78] has had a dramatic effect on antisense as well as tomato physiology research.

The advantages of antisense applications were also shown recently in the floral industry when a variety of flower patterns was achieved in *Gerbera hybrida* [79] and a white-flowering variety in *Dendranthema grandiflora* (florist's chrysanthemum) [80] after antisense chalcone synthase gene expression, sparked by the extensive investigations originally carried out in petunia [7,10,52].

Gene suppression by antisense RNA expression was shown to be useful in determining the role of

specific gene(s) involved in the signal transduction pathway of plant defense reactions. Clarence Ryan's group transformed tomato plants with a prosystemin antisense cDNA, causing complete inhibition of the systemic wound induction of proteinase inhibitors [81] in these transgenic plants as well as an increase in susceptibility to tobacco hornworm [82] demonstrating not only the involvement of the prosystem gene product in the signal transduction pathway but also that antisense technology can be used toward pest management.

Antisense transformation in *Nicotiana sylvestris* proved invaluable in identifying the physiological function of vacuolar class I β-1,3-glucanase gene (GLA). Transgenic plants expressing a portion of the coding region of GLA from tobacco effectively blocked specific expression of basic β-1,3-glucanase levels, both constitutively and upon induction in response to ethylene or infection with the fungal leaf pathogen *Cercospora nicotianae*, suggesting that expression of the isoform is not necessary for normal functions nor important in defense mechanisms [83]. Later findings show compensatory genes, not normally found in the leaves of tobacco and *N. sylvestris*, were induced upon virus infection either through an alternate pathway, alternate splicing or posttranscriptional modifications of known forms of β-1,3-glucanases, and strongly argues a role for involvement of enzyme activity in pathogenesis [84].

The potential to engineer seed oil compositions was shown when a seed-specific antisense stearoyl-acyl carrier protein desaturase gene, which plays a key role in determining the ratio of total saturated to unsaturated fatty acids, was expressed in *Brassica rapa* and *B. napus* (rapeseed), causing dramatic increases in stearate levels in the oil of the mature seed [85].

Flowering plants have evolved the mechanism of gametophytic self-incompatibility to prevent inbreeding as well as promote outcrossing. An antisense approach was utilized to regain self-fertilization in *Petunia inflata* by inhibiting an *S* allele protein of the pistil known to be a ribonuclease [86]. The transgenic plants failed to reject pollen of the same genotype. Likewise, nuclear male sterile mutants are common among many species of plants. Genetic male sterility can be brought about by genetic engineering where expression or repression of a gene involves phenotypic alterations in floral or reproductive tissues such as the destruction of tapetal cell layers, depletion of the flavonoid pigments, and expression of the *rolC* gene of the T-DNA of *Agrobacterium rhizogenes*, which are dominantly inherited [87]. Dominant male sterility is advantageous in hybrid seed production, but it is necessary to reverse this trait in the hybrids where the fruit is the product of harvest. Successful restoration of *rolC*-induced male sterility was achieved by expressing an antisense *rolC* gene in tobacco plants, thus creating a means of inducing and reversing male sterility in plants.

4.6. Unraveling plant development

The use of antisense sequences to manipulate the expression of endogenous plant genes seems promising for studies of plant development. Overexpressing antisense RNA of the *Arabidopsis* ankyrin-repeat (AKR) containing gene (more than one copy) caused a chlorotic phenotype resulting from reduced chlorophyll, carotenoid and internal membrane development in the chloroplasts, indicating an involvement of the AKR gene in the regulatory process of chloroplast differentiation [88].

Four auxin-regulated genes were studied during the growth cycle of a tobacco synchronized cell culture [89] by using an antisense construct to suppress one of the mRNAs. Since no effect on auxin-induced cell division was noted there remains no conclusive evidence of a role for these genes in cell division, although this had been previously suggested since they are transiently induced in an auxin-dependent manner during the transition from the quiescent phase to the synthesis phase of the cell cycle.

The potential of organ-specific modulation of a gene using antisense constructs was confirmed when leaf-specific inhibition of GUS gene expression was noted in transgenic tobacco plants expressing a 41-base homologous region spanning the translation start site of the GUS gene fused, in the reverse orientation, to the chlorophyll *a/b* promoter of *Arabidopsis* [90].

4.7. To ascertain transcript/protein relationships

Antisense strategies can be used to identify the

role of RNA sequences as well as the relationship between transcript and protein levels. The involvement of AU-rich sequences in plant intron splicing [91] and pre-mRNA splicing and intron removal from precursor mRNAs [92] was determined by expressing antisense clones of those regions in tobacco protoplasts and transgenic potato. To test whether local complementarity between viral RNA molecules can promote recombination in brome mosaic virus, antisense sequences were used to direct crossovers at or near the site of hybridization [93]. When steady-state levels of mRNAs for the polypeptides of the oxygen-evolving system, as well as the Rieske iron/sulfur protein, were suppressed in tobacco via overexpression of homologous antisense cDNAs, little effect on the corresponding thylakoid membrane protein levels was noted [94]. In addition, normal plant development progressed without a detectable enhancement of oxygen evolution, suggesting that these proteins are regulated posttranscriptionally.

Antisense RNA was able to efficiently inhibit the formation of the 10 kDa polypeptide of photosystem (PS) II in transgenic potato plants but had no effect on other PS II associated polypeptides, implying that transcripts are the rate-limiting step for the specific protein accumulation but not for the overall complex function [95]. Coordination of biosynthetic activities between nuclear and organelle gene expression of ribulose bisphosphate carboxylate (Rubisco) was examined by expressing antisense DNA sequences of the small subunit (SS) encoded in the nucleus [96]. Transgenic tobacco plants with suppressed *rbs*S mRNA and SS protein levels had no effect on the large subunit (*rbs*L) mRNA but caused a reduction in LS protein encoded on the chloroplast chromosome, providing evidence that the *rbs*S transcripts are the rate-limiting component of the multimeric protein complexes.

4.8. Novel uses of antisense gene expression

Höfgen et al. [97] have proposed using an antisense glutamate-1-semialdehyde aminotransferase gene, which decreases chlorophyll synthesis and causes a wide variety of chlorophyll variegation patterns, as a phenotypic assay for studies on the enzymes' role in the chlorophyll pathway.

Antisense RNA methods fit into the conditions needed to provide a positive-negative selection system for gene targeting in plants [98]. In a model system, the *nptII* gene in the sense orientation was placed within the genomic flanking sequences and its antisense was positioned outside the homologous region. Upon transformation, if random or non-homologous integration occurs, the plants become sensitive to kanamycin since the antisense gene is incorporated into the genome. If the sense gene is directed by the homologous sequences to be incorporated in the genome, the plant will be resistant to kanamycin and thus a single selection would yield a homologous recombination. Linkage of the anti-*nptII* gene to other gene sequences would provide a negative selectable marker in higher plants.

Incorporating a catalytic domain of hammerhead RNAs into DNA that encodes a target RNA would extend the utility of antisense technologies. A construct of the target cDNA is modified to contain a catalytic motif whose sequences are also part of a restriction recognition site found in the cDNA. In this manner all other sequences which flank the ribozyme are complementary to the cDNA. Transcription of the initial cDNA construct and the modified in opposing directions generates substrate (target) and antisense (ribozyme) RNAs. The RNA transcripts containing the catalytic ribozyme domain will cleave the corresponding complementary target transcript. Depending on the cloning, sense or antisense RNAs could be transcribed which contain the ribozyme. This could be of particular value against plant RNA viruses [99].

Numerous genes, whose products have important roles in plant growth and development and whose modifications would lead to insights into these processes or to improvement in the economic value of plants, are functionally redundant and hence difficult to manipulate genetically. Antisense strategies could provide a powerful means to circumvent these problems, eventually becoming invaluable as a tool for plant genetics.

5. Attenuation of plant viruses

A novel approach for enhancing resistance to plant viruses utilizes the expression of antisense RNAs directed against essential viral genes. It was

suggested that antisense RNA could function to inhibit virus replication since the genomes of plus strand RNA viruses have structural similarities to cellular mRNA and function as such during viral replication [100]. Since hybridization of antisense/viral RNA would occur over a relatively long stretch of nucleotides, the possibility of mutations in the viral populations is much reduced. The practice of cross-protection has been used with limited success for many years in traditional plant breeding programs to reduce crop losses to viral pathogens. This strategy was employed in transgenic plants with the expression of viral coat-proteins to provide an alternative version of cross protection [101,102]. Antisense technologies have been incorporated into the coat-protein theories of viral control but with little success [100,102–106]. Tobacco plants expressing the antisense RNA of the potato virus X coat protein [106] and the cucumber mosaic virus coat protein [102] only gave protection at very low inoculum concentrations. In other studies, protection was generally observed in the form of delayed resistance [103]. It may be that most RNA viruses replicate in the cytoplasm which may not be the site of antisense RNA function. In similar efforts, expression of satellite RNAs from plant viruses has caused some reductions in symptoms from satellite-free viruses [107,108].

An instance where an antisense construct directed against the coat protein gene was equally effective at mediating resistance as the sense construct was shown with potato leafroll virus (PLVR) in transformed Russet Burbank potato plants [109]. The effectiveness of this resistance was attributed to the fact that PLVR is confined to the phloem of the host plant (where the CaMV 35S promoter is known to be very active) where it accumulates slowly and generally maintains lower titers than other plant viruses. This should prove beneficial at the commercial level to affect reduction of PLVR in the field, since transmission of the virus by aphids within one field and between fields is considerably reduced when PLVR titers are low. Protection in the transgenic potato plants expressing the sense PLVR coat protein sequence was noted at comparable levels of expression of antisense PLVR RNA suggesting a similar mode of action and hinting that both positive and negative transcripts may be interacting with the opposite sense RNA strand of the virus [109].

A 15 to 30-fold decrease in systemic accumulation of tobacco mosaic virus (TMV) was noted in tobacco plants transformed with an antisense RNA construct directed at the 5′ untranslated leader segment of TMV, while the production of viral RNA was inhibited by as much as 25 to 50-fold [110]. Gonsalves and co-workers [111] transformed tobacco plants with sense and antisense untranslatable nucleocapsid protein (N) gene coding regions generated through primer-directed mutagenesis using RT-PCR of the lettuce isolate of tomato spotted wilt virus (TSWV). Both constructs provided protection against TSWV homologs and closely related isolates but not against more distant *Topoviruses*. Interestingly, greater protection was observed in transgenics that synthesized low levels of the N gene transcript (sense or antisense) which were shown to inhibit viral replication. The sense N gene RNAs provided greater protection than the antisense RNAs which is likely due to the sense RNA hybridizing to the positive strand genomic RNA before viral gene replication begins, while the antisense RNA can only hybridize to its complementary strand (the negative strand), which is replicated from the viral genomic RNA only after replication has been initiated. The S RNA segment of the TSWV genome has an ambisense gene orientation. Using protoplasts it was possible to demonstrate that cells expressing the antisense constructs did in fact support low levels of viral replication, delineating a cause for loss of protection at high inoculum concentrations. Similarly, transgenic tobacco plants expressing antisense sequences of the tobacco etch virus potyvirus (TEV) coat protein gene showed attenuated disease symptoms when compared to plants expressing untranslatable sense transcripts which did not develop symptoms, independent of inoculum levels or plant size. The resistance effect was shown to be mediated by an RNA molecule and due to decreased levels of virus replication [112].

Resistance to tomato golden mosaic virus (TGMV) in greater than 90% of the transgenics from a tobacco plant line was achieved by designing a genetic cassette containing antisense of DNA sequences required for TGMV DNA replication

fused to the hygromycin resistance gene. This fusion allowed easier selection of transgenics carrying the antisense gene. TGMV, a single stranded DNA virus, is known to replicate in the nucleus where the opportunity for duplex formation or interaction with the antisense RNA may be greater [113]. Using this same DNA sequence, known as the AL1 gene, which is conserved in other geminiviruses, the authors were able to test its capability to provide cross protection type resistance to two other geminiviruses [114]. When the transformed plants were challenged with beet curly top virus (BCTV) a 4-fold reduction in viral DNA accumulation was noted compared to no reduction when infected with African cassava mosaic virus (ACMV). Both have similar regions of homology with TGMV DNA, but BCTV contains a 280 nucleotide stretch of 80% homology, suggesting that complementation over a critical region is necessary to provide the antisense effect. When the BL1 gene from TGMV, thought to be involved in virus movement, was used in the sense and antisense orientation to replace the ACMV coat protein coding sequences and co-inoculated with ACMB DNA B, systemic spread of ACMV in *Nicotiana benthamiana* transformed plants was inhibited by the sense but not the antisense constructs [115]. The important findings established here are that the potential for developing multifunctional antisense cassettes for viral control does exist.

Huntley and Hall [116] in their studies on brome mosaic virus replication in barley protoplasts showed that targeting of the viral minus strand promoters with sense and antisense transcripts reduced progeny viral RNAs, in some cases, by more than 90%.

The results obtained thus far in suppressing viral functions in plants are very promising and suggest that antisense techniques will be useful in developing alternative methods for resistance to plant viruses.

6. Attaining the antisense effect

Levels of antisense suppression can vary widely within a population of transgenic plants and have been attributed to aspects which include position effect, rearrangement of the target gene, duplicated copies of the antisense sequences, the capability of the promoter, and the specificity of the antisense sequence for its target RNA. This taken into account, it has been demonstrated that a correlation between the level of phenotypic inhibition and the antisense transcript accumulation or antisense gene copy number does not always exist.

6.1. Antisense promoters

In vitro studies have alluded to the fact that a high ratio of antisense to sense RNA is necessary to attain effective suppression of the target gene [6], therefore most studies with foreign gene expression in plants utilize a stronger promoter for the antisense transcript than that for the target gene [5,7,117,118]. Studies in the field have widely exploited the Cauliflower mosaic virus (CaMV) 35S RNA promoter with foreign genes such as nopaline synthase (*nos*) [5], chloramphenicol acetyltransferase (CAT) [6], bialaphos (*bar*) [119] and β-glucorindase (GUS) [120,121] as well as with endogenous antisense gene sequences of β-1,3-glucanase [83,84], the tomato polygalacturonase (PG) gene [25,58], auxin-regulated genes from tobacco [89], the ethylene forming enzyme from tomato [59], tomato pectin esterase gene [76], the intronless coding region of a maize zein storage protein gene [92], chalcone synthase genes [7,53,79,80,122], and the cucumber mosaic virus coat protein gene [102] just to name a few. Other promoters such as those from the chlorophyll *a/b* binding protein gene [90,118], the *nos* gene [6,111] the CaMV 19S [117], the potato granule-bound starch synthase gene [123], the carrot phenylalanine ammonia-lyase (PAL) gene [6], the petunia chalcone synthase promoter [52,122] and the promoter from the soybean β-conglycinin storage protein gene [64] have also been used.

Expression of CHS antisense resulted in an altered flower development pattern in tobacco and petunia plants [7]. The phenotypes exhibited correlated with a reduced CHS mRNA level in the flowers and variation in spatial distribution of the antisense gene expression in the same and different flowers [124]. In evaluating the efficiency of these CHS antisense genes, it was determined that the antisense CHS genes driven by either the CaMV 35S RNA or the endogenous CHS promoter were probably not expressing high enough levels of

transcripts to provide an excess to target the CHS mRNA [122,125].

Identical promoters and terminators were employed with a simple inversion of the GUS gene coding sequence to yield sense and antisense constructs. To eliminate any differential developmental and tissue-specific expression from the use of two promoters, only the CaMV 35S RNA promoter was utilized. In the transformed tobacco plants examined, which were shown to contain 1–5 copies of the antisense gene, suppression of GUS was consistently greater than 90% relative to the wild type activity, establishing that a target gene driven by a strong promoter can be almost completely suppressed [121].

Several plasmids were constructed with the sense and antisense of the CAT gene linked to various promoters: *nos*, CaMV 35S RNA and the phenylalanine ammonia-lyase (PAL) gene promoters, and utilized to transform carrot protoplasts [6]. The *nos* and CaMV promoters gave similar levels of inhibition, but when the PAL promoter was used, a reduction in inhibition was noted meaning that (at least with the CAT gene) the level of antisense effect correlates to the promoter strength.

Transgenic tobacco plants were found to contain *rbc*S antisense transcripts in root tissues, which do not synthesize the small subunit SS, at levels comparable to those in leaves, proposing that inhibition of Rubisco, an extremely abundant protein, does not require an excess of antisense mRNA [31].

It would be invaluable to offer a degree of flexibility in gene regulation via antisense strategies. Some investigators have proposed the use of heat inducible promoters to reverse the repressive effect upon induction at elevated temperatures [126] or alternately express the sense or antisense constructs upon induction [127]. Recently, expression of an antisense CHS gene in anthers was possible with a modified CaMV 35S RNA promoter containing inserted anther box sequences. An inhibition of pigment synthesis in anthers resulted and subsequently led to male sterility, implicating that flavonoids may play a role in male gametophyte development [128], but also illustrating that antisense specific cell-type expression can be accomplished in plants.

To date, most reports of foreign and endogenous gene regulation in plants have made use of RNA polymerase II promoters [60,82,117], particularly the strong CaMV 35S RNA promoter [6,7,56,89,90,102,104,105,118–120], to generate antisense RNAs. The inability to express sufficient levels of antisense RNAs in the cell has often led to incomplete and ineffective gene regulation [122,125] and this has been particularly true with attempts to control viral infections in plants [102,105].

The use of polymerase III promoters provides an alternative approach for producing high levels of antisense transcripts [129]. The transcripts produced by RNA polymerase III are neither capped nor polyadenylated and since the genes themselves are very small, in general, the products are substantially shorter than those transcribed by RNA polymerase II. These smaller transcripts may impart an advantage both in accessibility and in hybridization to target sequences [129]. The high degree of sequence conservation of polymerase III promoters and their known activity in all cell types provides another advantage for their use [23]. A tRNA structure attached to the end of the antisense sequence may also aid in stability and affect cellular localization [130] which should, in turn, influence its effectiveness as a suppressor of gene function.

Bourque and Folk [129] reported that fusing a tRNA methionine gene from soybean to CAT sequences effectively inhibited CAT activity in carrot protoplasts five fold more than the CaMV 35S RNA promoter. These results established that antisense sequences transcribed by RNA polymerase III efficiently suppressed gene expression in plant cells. This is supported by more recent results in transgenic tobacco plants (unpublished data). Investigations into tRNA genes isolated from *Arabidopsis* and soybean are being evaluated for their ability to transcribe sequences involved in endogenous gene suppression and for the control of viral infection in plants (unpublished data).

6.2. Heterologous vs. homologous duplex formation

The degree of homology and, perhaps more importantly, the specific region of homology between the target and antisense RNA may be a determining factor in how these two transcripts associate in

the cell and possibly dictate the mechanism by which the regulation is affected [33]. Assuming that an RNA double helix is thermodynamically stabilized by the presence of terminal unpaired bases [131] then it is probable that heterologous duplexes would be more stable by sustaining a reduced rate of mRNA degradation compared to a homologous duplex [33]. Transgenic potato plants expressing the waxy gene, which encodes a granule-bound starch synthase isolated from cassava, were able to suppress the synthase and showed reduced amylose content as well, confirming that heterologous genes can be used to achieve antisense effects [123]. When intron sequences are included in the construct in a homologous system [132], the antisense effect were still accomplished.

A heterologous antisense cucumber NADH-hydroxypyruvate reductase RNA used to modulate activity of its corresponding enzyme in tobacco was not able to generate a reduction below 50% of the endogenous protein levels, possibly owing to the heterogeneity of the RNA interaction [33]. These results hint at translational interference from the duplex molecule since the antisense RNA clearly showed no effect on sense RNA levels. Likewise, a glutamine synthase antisense RNA in tobacco failed to affect the steady-state level of the native target mRNA [133]. If it is believed that an RNA/RNA duplex can have a negative effect on translation efficiency, then it would be reasonable to expect that any association of the endogenous RNA with the ribosomes would likewise be diminished resulting in a destabilization of the target RNA [33].

6.3. Gene dosage, position effect and methylation

In transformation experiments, insertion of the transgene into a particular site is a random event thought to affect subsequent expression which ultimately results in a high degree of variability in inhibition levels seen in a population transformed with an antisense gene. Introducing more than one copy of a complementary gene does not necessarily result in increased expression of the antisense gene and down regulation of the target gene.

Variation associated with the site of insertion but not copy number was used to explain the range of 50–95% down regulation of polygalacturonase (PG) enzyme activity [72] since it was shown that the antisense DNA was present as a single copy per diploid genome. The progeny from selfed plants containing the antisense in two copies exhibited a further decrease in PG expression, to a level of 99% reduction of the wild-type PG, unquestionably pointing to the effect of gene dosage in the homozygous compared to the heterozygous plants. In another study, a greater antisense effect was also associated with copy number duplication in the progeny implying the presence of a homologous antisense PG gene in tomato [68]. Hall et al. [76] did not note an enhanced reduction in pectin esterase (PE) activity in tomatoes homozygous for the antisense PE gene as was seen in other studies on PG activity [25] and ethylene synthesis [60].

The progeny analysis of tomato plants expressing a PME antisense gene showed a decreased endogenous enzyme activity, but the homozygous progeny had levels of PME activity similar to that noted in the heterozygous plants even though the steady state level of the antisense RNA in homozygous plants was higher than that in the heterozygous plants [74]. This undoubtedly shows that no further reduction of the enzyme activity was attained with an increase in antisense mRNA levels. As expected, the selfed plants that segregated out the antisense gene had increased PME activity.

Expression of an antisense gene of maize granule-bound starch synthase (GBSS) resulted in a 20–90% inhibition of potato GBSS activity in tubers while the GBSS sequences, isolated from potato and expressed as antisense RNA, in that same system caused an 80–100% suppression. The copy number effect could only be noted with the heterologous constructs. In both of these experiments the GBSS protein accumulation correlated with the enzyme activity level [134,135].

In a similar study, potato plants transformed with GBSS in the antisense orientation showed a range of suppression from complete inhibition to no detectable effect although it had been shown that each plant tested contained from 1 to 5 inserts [123]. A 10 kDa polypeptide of photosystem II was efficiently suppressed in transgenic potato plants by an antisense construct. While the steady

state level of the antisense mRNA was approximately 1% of the wild type level there was no mutual relation between the level of antisense mRNA and the level of reduction in the sense transcript [95].

In studies using antisense RNA to target the tobacco thylakoid membrane protein it was noted that sense mRNA levels were modified by the presence of the antisense RNA but little influence was observed on the level of corresponding protein [94]. While genetic analysis showed no link between phenotype and chromosomal position of the CHS antisense gene in transgenic petunia plants, position effect relative to the target gene was not ruled out [53]. However, there was some association observed between antisense gene dosage and Rubisco levels in transgenic tobacco plants [41,44].

Transgenic petunia plants exhibited no correlation between the number of antisense chalcone synthase (CHS) gene copies inserted and the steady-state level of antisense RNA [7], confirming the observation that gene expression is influenced by relative positions of the sense and antisense genes. Furthermore, several transformants exhibiting the same level of antisense RNA gave a wide array of changes in flower pigmentation, implicating that sequences adjacent to the site of insertion have a quantitative and qualitative influence on antisense gene expression. Surprisingly, some transgenics with very low antisense RNA levels were able to produce very extreme phenotypic effects [7].

Some differences in gene expression within a transformed population have been correlated to differential methylation. However, van der Krol et al. [53] found no cytosine methylation at a sensitive site in the CaMV 35S RNA promoter used to transcribe the antisense CHS gene.

7. Target sequences

Each biological system appears to respond differently to antisense strategies. It has generally been accepted in prokaryotic and animal systems that antisense RNA directed at the 5′ region of a gene was most effective in suppressing gene expression, but there is no general agreement in the literature as to what regions or lengths of subgenomic fragments serve as the optimal antisense sequences. Some reports have shown antisense RNA directed against the 5′ leader and the 3′ trailer of mRNAs to be particularly effective [136,137], while others report that antisense oligonucleotides complementary to splice junctions inhibit transcript accumulation [138]. Several groups [117, 118,120,125] have experimented with expressing various regions of the target genes as antisense sequences in plant systems, but again no consensus has been established.

A relative comparison of the effectiveness of chalcone synthase (CHS) subgenomic fragments in petunia illustrated that RNA complementary to the 3′ portion of the CHS gene was more effective at affecting flower pigmentation than that cloned from the 5′ portion of the gene [52,122], probably due to a lower intrinsic stability of this RNA. Further evaluation of the 3′ segment divided into two smaller fragments resulted in a visible effect on flower pigmentation only from the shorter 5′ most region, giving evidence that sequence length of the antisense RNA may contribute to the effectiveness of the inhibitory effect [122]. No specific sequence could be identified that appeared to be associated with the repressed state.

Utilizing the nopaline synthase (*nos*) coding region, antisense plasmids containing a full length, a 5′ segment, three internal segments and about one-half of the 3′ region were constructed and linked to the promoter of a petunia chlorophyll *a/b*-binding protein gene [118]. The sequences from the 3′ half of the *nos* transcript were the most effective, even more so than the full length coding region, showing that various regions of a coding sequence in the opposing orientation can all have a positive effect on gene inactivation but, depending on the fragment sequence or length, the level of suppression can vary. Rothstein et al. [5] cloned only approximately 2/3 of the 5′ portion of the *nos* gene in the reverse direction and was able to reduce activity in transformed tobacco plants from 10 to 50-fold.

A construct which generated transcripts containing the 5′ terminal 172 bp of the CAT gene in the antisense direction was found to be less effective than the full length CAT gene sequence in

transgenic tobacco plants [117]. In another study [129] using several fragments of the CAT gene coding sequence fused to a soybean tRNA methionine gene, it was determined that a 341 bp segment from the 3′ portion of the CAT gene was an effective construct while a 5′ region of 154 bp was not as efficient at suppressing CAT activity in carrot protoplasts. Two other 5′ fragments of 214 and 391 bp were not capable of suppressing gene expression and were likewise shown to be inefficiently transcribed in an in vitro transcription assay using heterologous cell extracts known to support polymerase III transcription.

To determine which region of the GUS gene was responsible for inhibition, plasmids containing various sequences of this gene were cloned in the antisense orientation [120], including a full length sequence with and without a promoter, three 5′ terminal sequences (with 647, 583 and 68 bp of antisense) and a 3′ terminal sequence (1239 bp of antisense) linked to the CaMV 35S RNA promoter. The full length sequence with a promoter gave a 60–70% inhibition as did the two 5′ terminal constructs of longer lengths. The 5′ construct with only 68 bp of antisense sequence and the promoter-less full length antisense gene were completely inactive, the latter result confirming that antisense transcription is necessary for the inhibitory effect. The 3′ terminal construct containing 1239 bp was essentially ineffective, giving extremely variable results. When this construct was portioned into two smaller fragments, the central region of the sequence was shown to be more efficient than those from the extreme 3′ end. It was denoted that the difference in levels of inhibition brought about by the various fragments, especially with those containing the same sequences, could be accounted for by changes in alterations of stability and possibly the secondary structure of the antisense RNAs.

8. Mechanisms of action

Despite the amount of literature that has amassed on this system of gene regulation, there are few studies which have directly addressed mechanisms or modes of action in plant systems. From studies with endogenous gene suppression in plants, one overriding theme that is generally noted is the accompanying reduction in target mRNA accumulation when antisense RNA is present [90,117]. Below is a collection of studies which are categorized according to individual theories on how antisense RNA functions in the cell at the molecular level (summarized in Table 2).

8.1. Transcriptional/posttranscriptional control

If antisense RNA directly affected transcription,

Table 2
Theories on antisense mechanisms in plants

Transcriptional/posttranscriptional control	Reference
1. Transcription rates of antisense and target gene are unaffected	22a,60b,89
2. Levels of target mRNA correlate to phenotypic changes	61,103b,104
3. No excess antisense RNAs detected	51,105
4. Target transcript pool size is causal factor in down regulation	81,82
5. Transcription rate of target RNA same level in control and transgenes	84,89
6. Same level of antisense RNA gives varying degrees of inhibition	85,89,95,110b
7. Antisense RNA levels same in tissue expressing and devoid of target RNA	81,82,105
8. Introns not processed in coding regions of antisense genes	103,108
Translational control	
1. Levels of antisense transcripts correlate to degree of inhibition	101
2. No change in steady state level of endogenous mRNA	18
3. Lower target protein synthesis correlates to reduced target mRNA	18
4. Total and ss RNA preps yield same level of target RNA	18
5. Antisense oligos can inhibit in-vitro translation	20

the effect would be through an interaction with the DNA template or the nascent sense transcript, which would more than likely involve premature termination. There is no evidence to support either one of those interactions and since it has frequently been reported that the transcription rate of the antisense and the target gene are unaffected by expression of the antisense sequences, this suggests that the repressive effect is functioning post-transcriptionally [58,140]. It has been postulated that if transcript abundance were not the rate-limiting step, the inactive state may not be noted [94].

Antisense transcripts could affect transcription at the level of mRNA processing by interfering with splicing of introns during the maturation of the mRNA [124]. Two introns contained in the pectin methylesterase gene [74], when placed in the antisense orientation, were not processed. The same introns were excised when the construct was oriented in the sense format, also providing evidence for posttranscriptional regulation.

Early theories proposed that an RNA duplex between the antisense RNA and the target RNA was the initial causal factor contributing to the repressed effect. It has further been hypothesized that such a double stranded RNA molecule is unstable and an easy target for degradation by nuclear RNases. Investigations looking in earnest for the duplex formation have continually come up without any evidence. Steady-state levels of target chalcone synthase (CHS), but not the antisense, mRNA correlated well with the degree of changes in floral pigmentation, indicating that perhaps a combined degradation of the mRNAs occurred after duplex formation [52,53]. But even in the transgenic plants with the most extreme phenotype, white flowers in petunias, no excess of antisense CHS mRNA was detected [140]. No duplex formation was observed in any of the transformants and often no antisense CHS RNA was present, implying that RNA duplex formation might not be the sole mechanism for the observed down regulation of the CHS gene. Furthermore, CHS mRNA isolated from these white flower buds was totally susceptible to RNase A digestion suggesting the absence of RNA duplex formation between the CHS sense and antisense RNAs [53].

Other research which has addressed the mechanism of antisense regulation in plants comes from studies involving suppressing of ribulose bisphosphate carboxylase (Rubisco), the major regulatory enzyme of carbon metabolism, as an alternative approach in the study of nuclear-chloroplast interactions in photosynthesis [31,96]. Expression of antisense DNA sequences from the small subunit (SS) of Rubisco gave rise to drastically reduced levels of SS mRNA and protein, as well as the protein from the large subunit (LS) but not the mRNA from LS, suggesting that regulatory mechanisms exist between two compartments, the nucleus and the chloroplast where SS and LS are encoded respectively. It appears that *rbc*S transcript pool size is the determining factor regulating Rubisco levels. Pulse-labeling experiments indicated that the *rbc*L mRNAs were translated with about the same efficiency in the transgenic and the control plants, implicating posttranscriptional regulation. From Northern data, the *rbc*S mRNA antisense transcripts were noted to accumulate to low abundance, as with the CHS system described previously, with levels varying between transformants. Results marked no preferential degradation of antisense mRNA in target gene tissues (leaves) since comparable antisense RNA levels were observed in root tissues which do not synthesize endogenous *rbc*S mRNA.

Rothstein et al. [5] used the *nos* promoter to transcribe the sense *nos* sequences and the CaMV 35S RNA promoter to drive the *nos* antisense sequences in transformed tobacco plants. Analysis of the steady-state levels of transcripts by RNase protection assays indicated that the antisense RNA level was approximately equal to the level of *nos* mRNA in control plants. An antisense mechanism was proposed where double-stranded RNA is degraded more rapidly than the unhybridized RNAs, since it appears that suppression is working primarily through a decline in the sense mRNA level.

Sheehy et al. [58] noted that tomato transformants that had very different levels of antisense RNA all exhibited approximately the same drop in the level of polygalacturonase (PG) enzyme and that the synthesis of the endogenous PG mRNA was not totally eliminated, suggesting a post-

transcriptional mechanism for down regulation. This idea was further strengthened by nuclear run-off experiments illustrating that PG mRNAs are transcribed at the same rate in transgenic and control plants, but antisense PG mRNAs are transcribed at a higher rate. It could be postulated, that as quantities of antisense mRNA accumulate, that the duplex formed with the sense mRNA is continually degraded and unable to accumulate and consequently very little PG protein is made [31]. In other tomato fruit ripening experiments, the reduction in the level of PG mRNA noted occurs concomitantly with a reduction in PG activity, indicating that antisense RNA cannot simply inhibit mRNA translation [72].

From the above data, it seems plausible that the antisense RNA may be functioning at different levels to attain silencing. In systems where endogenous antisense transcripts occur, the role of such a complementary strand is speculated to be at the posttranscriptional level where regulation is probably due to alterations in mRNA stability [30]. Since foreign genes used to transform plants do not contain 5′ leader sequences, it is unlikely that the inactivation is occurring at the site of translation initiation [7]. Direct evidence of interactions between sense and antisense RNAs comes from studies aimed at viral RNAs encoded in the cytoplasm [102,105,106].

Hamilton and Grierson [141] contend that if a duplex is formed between the sense RNA and the antisense RNA and subsequently degraded, then both genes would be suppressed, not only the sense gene. They propose that only a brief, partial interaction occurs between the two transcripts, preventing the sense primary transcript from maturing, implying that the fate of the antisense transcript is independent of the sense transcript.

8.2. Translational control

There have been some reports where the mode of action for antisense control has implicated cytoplasmic inhibition noted by a reduction in the translation rate of the target sequence either with or without a concomitant decreased accumulation of its transcript. In contrast to nuclear inhibition, where it is proposed that degradation of a duplex RNA occurs, these results suggest a stable duplex formation which is transported, ultimately causing an interference with the translation machinery.

The antisense alfalfa glutamine synthetase (GS_1) transcript was able to suppress GS_2 in tobacco, to which it showed a lower degree of homology, suggesting that duplex formation over a short sequence length is sufficient for effective down regulation [133]. Although an accumulation of high levels of GS_1 transcripts was observed and could be correlated to the degree of inhibition of protein synthesis, there appeared to be no change in the steady-state level of GS mRNA from the endogenous gene. This would imply that the RNA heteroduplex would be stable, acting like RNA molecules with extensive secondary structure, and that inactivation is occurring at the translational level either through blocking of ribosome binding or transport out of the nucleus.

Both RNA level and translation efficiency were reduced in transgenic tobacco plants expressing the antisense bialaphos (*bar*) resistance gene which encodes the phosphinotricin acetyl transferase (PAT) protein [119]. Plants expressing the antisense transcript showed a 13-fold lower protein synthesis of PAT when compared to wild type levels and was proportional to the reduced accumulation of *bar* mRNA, but not the antisense mRNA. The rate of turnover in *bar* RNA, from inhibitor experiments, was noted to be the same in control and transgenic plants [31]. Preparations of total RNA contained similar quantities of *bar* RNA when compared to single-stranded (ss) RNA preparations, meaning rapid degradation and low accumulations of any duplexes that were formed. If it is assumed that the major portion of the ss *bar* RNA detected represents cytoplasmic RNA, then the reduction in protein synthesis is probably due to translation interference, most likely caused by unstable base pairing rather than a hinderance of transport from the nucleus.

Two oligodeoxynucleotides complementary to sequences of the 5′ end of the p126 gene of tobacco mosaic virus (TMV) were able to inhibit in vitro translation of the genomic RNA and this effect was attributed to a direct interference with ribosome function [142].

9. Perspectives

It is obvious that the use of antisense DNA or RNA is a very useful strategy in the down regulation of genes in plants. Replete examples exist in the literature where antisense sequences have been successful in achieving partial or complete inactivation of target genes signifying that this technology has a broad range of utility. But to more effectively develop the use of antisense RNAs for plants, a variety of questions still need to be addressed, namely mechanisms. Probably the most overriding question is 'Can attaining the antisense effect in plants be standardized so that it becomes a routine method?'. It would be ideal to be able to draw upon observations of complementary sequences that exist naturally in plant systems, but the yet undefined role of these transcripts excludes that route. Therefore, if we are to reach such a goal, many factors, suspected to have a consequence on the inhibitory level, will have to be tackled.

Does the antisense transcript need to present at 'super'-stoichiometric levels? One area of antisense RNA design that could easily lend to optimization is the choice of promoters. Inducible promoters might serve a useful role [126,127], particularly where a null mutation would cause a disruption of normal cellular growth and development. On the other hand, the use of truly constitutively expressed promoters [129] would be fitting for those situations, such as viral gene regulation, where the appearance of the target gene cannot be controlled. Since it is thought that the antisense effect is very specific, continual expression could easily be tolerated by the plant system. The use of strong promoters [5,122] is thought to be the best method for achieving high levels of antisense expression, although some reports have indicated that only minimal amounts of antisense RNA were required to inhibit target genes, even if abundantly expressed [31,121]. The use of chimeric promoters consisting of structural RNAs, such as the tRNA genes [129], may provide the necessary secondary structures required for both biological activity and increased stability.

What is the most effective target sequence? When constructing the antisense gene, the degree of homogeneity to the target gene, the length of the sequence strand and the regions contained within, which are perhaps required for association with other molecules, are more difficult considerations. In some eukaryotic systems, designing an antisense RNA where the 3′ end would conceal the 5′ cap structure and thus prevent ribosome association, would seem feasible. But, in plant systems, where bacterial reporter genes have been down regulated without the necessary 5′ leader region, it can be reasoned that inhibition is probably occurring in the nucleus. The many different possible modes of action for inhibition in the nucleus complicates antisense RNA design, but the overall effort (whether using 3′, internal or 5′ sequences) is presumably forcing a secondary structure that restricts the availability of the target sequences for subsequent association or binding. Determining the length of that sequence probably depends on the critical nature of RNA duplex degradation by specific RNases. The percent homology between the antisense and target sequences may contribute to the stability of the heteroduplex, where it has been suggested that a degree of heterogeneity may be advantageous [133]. Factors which may be definitive in calculating the critical sequence length or content and of which no substantial data is yet available are those such as the potential secondary structure, the GC content, or the protein binding sites, which may all play an important role in the efficiency of the regulatory effect.

Is the relative position of the antisense transgene to the target sense gene a factor in the efficiency of suppression? As mentioned earlier, the random nature of antisense transgenes leads to a wide variety of levels of inhibition. Increasing the copy number can give negative or positive effects with regards to the degree of inhibition noted [123]. Where no effect has been observed with an increase in gene copy number [7], it has been speculated that relative positioning of the transgene is the ultimate influencing factor upon regulation. Flanking sequences have also been implicated in affecting the qualitative and quantitative influence on gene expression [7]. The overall variability within plant systems complicates com-

parative analysis, either between antisense genes, promoters or even the repressive effect in different tissues. It might be useful to keep in mind that, as Matzke et al. [9] pointed out, the most consistent and stable expression in plants has been derived from those constructs where repeated elements were lacking and single copies of transgenes were cloned.

Although it is apparent that the concentration, stability and localization of antisense RNA within the cell is important for its activity, it is still unclear at what level antisense RNA must act to be most effective. Many different theories have been proposed for the mechanism of action of the antisense RNA in the cell, suggesting that perhaps more than one mode is functional or that suppression may work differently from one plant system to another or even between different genes in the same system. Where in the cell does the inhibitory effect take place, nucleus or cytoplasm, and at which point in the flow of information, from DNA template to the protein product, does the optimal effect result? Many observations noting that the transcription rate of the antisense and the target gene are unaffected by the expression of the antisense sequences have given rise to the theory that the repressive effect is functioning posttranscriptionally [58,140]. Are RNA duplexes formed between the sense and antisense transcripts and, if so, are they degraded by specific RNases? Since CHS sense expression gives an antisense effect, without the possibility of RNA:RNA duplex formation, there may be other mechanisms working [14] such as interference with the transcription process itself resulting from the antisense RNA interacting with the endogenous target DNA [122].

Does an unwindase activity as that noted in mammalian systems [143] also exist in plants? Very few studies have observed a reduction in expression specifically due to an interference with translation, but in those cases, it is possible that export of the proposed duplex into the cytoplasm was blocked. To further define whether the regulatory effect is occurring in the nucleus or the cytoplasm, more investigations are needed. One possible study to gain more insight might include restricting transport outside of the nucleus. Perhaps in depth studies with viral genes, where replication is known to occur in the cytoplasm, or analysis of antisense genes directed against mitochondrial genes known to be transported from the nucleus, would add to the overall knowledge.

But, for the time being, although tedious, each proposed antisense sequence will have to be empirically analyzed for its optimum length, homology to coding region and specificity of action to achieve the desired suppressive effect.

Acknowledgements

This work was supported by a grant from the Monsanto Corp., St. Louis, MO and USDA subgrant 92-34190-6941 from The Consortium for Plant Biotechnology Research, Inc.

References

[1] R.W. Simons and N. Kleckner, Translational control of IS10 transposition. Cell, 34 (1983) 683–691.
[2] P.J. Green, O. Pines and M. Inouye, The role of antisense RNA in gene regulation. Ann. Rev. Biochem., 55 (1986) 567–597.
[3] R.W. Simons, Naturally occurring antisense control — A brief review. Gene, 72 (1988) 35–44.
[4] J.G. Izant and H. Weintraub, Inhibition of thymidine kinase gene expression by antisense RNA: A molecular approach to genetic analyses. Cell, 36 (1984) 1007–1015.
[5] S.J. Rothstein, J. DiMaio, M. Strand and D. Rice, Stable and heritable inhibition of the expression of nopaline synthase in tobacco expressing anti sense RNA. Proc. Natl. Acad. Sci. USA, 84 (1987) 8439–8443.
[6] J. Ecker and R. Davis, Inhibition of gene expression in plant cells by expression of antisense RNA. Proc. Natl. Acad. Sci. USA, 83 (1986) 5372–5376.
[7] A.R. van der Krol, P.E. Lenting, J. Veenstra, I.M. van der Meer, R.E. Koes, A.G.M. Gerats, J.N.M. Mol and A.R. Stuitje, An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature, 333 (1988) 866–869.
[8] R.B. Flavell, Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA, 91 (1994) 3490–3496.
[9] M. Matzke and A.J.M. Matzke, Genomic imprinting in plants: Parental effects and *trans*-inactivation phenomena. In Ann. Rev. of Plant Physiol. Plant Mol. Biol., 44 (1993) 53–76.
[10] C. Napoli, C. Lemieux and R. Jorgensen, Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in *trans*. Plant Cell, 2 (1990) 279–289.
[11] D. Grierson, R.G. Fray, A.J. Hamilton, C.J.S. Smith and C.F. Watson, Does co-suppression of sense genes in transgenic plants involve antisense RNA? Trends Biotechnol., 9 (1991) 122–123.

[12] H. Vaucheret, J. Kronenberger, A. Lepingle, F. Vilaine, J.-P. Boutin and M. Caboche, Inhibition of tobacco nitrite reductase activity by expression of antisense RNA, Plant J., 2 (1992) 559–569.

[13] M.A. Matzke, M. Primig, J. Trnovsky and A.J.M. Matzke, Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants, EMBO J., 8 (1989) 643–649.

[14] A.R. van der Krol, L.A. Mur, M. Beld, J.N.M. Mol and A.R. Stuitje, Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression, Plant Cell, 2 (1990) 291–299.

[15] F.F. Assaad, K.L. Tucker and E.R. Signer, Epigenetic repeat-induced silencing (RIGS) in *Arabidopsis*, Plant Mol. Biol., 22 (1993) 1067–1085.

[16] M.A. Matzke, F. Neuhuber and A.J.M. Matzke, A variety of epistatic interactions can occur between partially homologous transgene loci brought together by sexual crossing, Mol. Gen. Genet., 236 (1993) 379–386.

[17] P. Meyer and I. Heidmann, Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants, Mol. Gen. Genet., 243 (1994) 390–399.

[18] T. Fujiwara, P.A. Lessard and R.N. Beachy, Inactivation of the nopaline synthase gene by double transformation: reactivation by segregation of the induced DNA, Plant Cell Rep., 12 (1993) 133–138.

[19] R. Jorgensen, The germinal inheritance of epigenetic traits and its possible significance in the evolution of plant chromosome organization patterns, Phil. Trans. R. Soc. Lond. Ser. B., 339 (1992) 173–181.

[20] F. deCarvalho, G. Gheysen, S. Kushnir, M. Van Montagu, D. Inzé and C. Castresana, Suppression of β-1,3-glucanase transgene expression in homozygous plants, EMBO J., 11 (1992) 2595–2602.

[21] N. Houba-Hérin and M. Inouye, Antisense RNA, in: F. Eckstein and D.M.J. Lilley (Eds.), Nucleic Acids and Molecular Biology, Springer-Verlag, Berlin, 1987, pp. 210–220.

[22] M. Inouye, Antisense RNA: its function and application in gene regulation — a review, Gene, 72 (1988) 25–34.

[23] K.M. Takayama and M. Inouye, Antisense RNA, Crit. Rev. Biochem. Mol. Biol., 25 (1990) 155–184.

[24] S.K. Kim and B.J. Wold, Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA, Cell, 42 (1985) 129–138.

[25] C.J.S. Smith, C.F. Watson, J. Ray, C.R. Bird, P.C. Morris, W. Schuch and D. Grierson, Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes, Nature, 334 (1988) 724–726.

[26] M.A. Rezaian and R.H. Symons, Anti-sense regions in satellite RNA of cucumber mosaic virus form stable complexes with the viral coat protein gene, Nucl. Acids Res., 14 (1986) 3229–3239.

[27] J.C. Rogers, RNA complementary to α-amylase mRNA in barley, Plant Mol. Biol., 11 (1988) 125–138.

[28] H. Akagi, M. Sakamoto, C. Shinjyo, H. Shimada and T. Fujimura, A unique sequence located downstream from the rice mitochondrial *atp6* may cause male sterility, Curr. Genet., 25 (1994) 52–58.

[29] G. Schmitz and K. Theres, Structural and functional analysis of the *Bz2* locus of *Zea mays*: characterization of overlapping transcripts, Mol. Gen. Genet., 233 (1992) 269–277.

[30] S. Dolfini, G. Consonni, M. Mereghetti and C. Tonelli, Antiparallel expression of the sense and antisense transcripts of maize α-tubulin genes, Mol. Gen. Genet., 241 (1993) 161–169.

[31] S.R. Rodermel and L. Bogorad, Antisense mRNA inhibition of ribulose bisphosphate carboxylase — the most abundant protein in photosynthetic cells, in: J.A.H. Murray (Ed.), Antisense RNA and DNA, Vol. 11, Modern Cell Biology, Wiley-Liss Inc., New York, 1992, pp. 121–135.

[32] M. Tabler, Antisense RNA in plants: A tool for analysis and suppression of gene function, in: K.A. Roubelakis-Angelakis and K. Tran Thanh Van (Eds.), Morphogenesis in Plants, Van Plenum Press, New York, 1993, pp. 237–258.

[33] M.J. Oliver, D.L. Ferguson, J.J. Burke and J. Velten, Inhibition of tobacco NADH-hydroxypyruvate reductase by expression of a heterologous antisense RNA derived from a cucumber cDNA: Implications for the mechanism of action of antisense RNAs, Mol. Gen. Genet., 239 (1993) 425–434.

[34] W.P. Quick, U. Schurr, K. Fichtner, E.-D. Schulze, S.R. Rodermel, L. Bogorad and M. Stitt, The impact of decreased Rubisco on photosynthesis, growth, allocation and storage in tobacco plants which have been transformed with antisense *rbc*S, Plant J., 1 (1991) 51–58.

[35] G.S. Hudson, J.R. Evans, S. von Caemmerer, Y.B.C. Arvidsson and T.J. Andrews, Reduction of ribulose-1,5-bisphosphate carboxylate/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants, Plant Physiol., 98 (1992) 294–302.

[36] R. Höfgen and L. Willmitzer, Transgenic potato plants depleted for the major tuber protein patatin via expression of antisense RNA, Plant Sci., 87 (1992) 45–54.

[37] M. Lauerer, D. Saftic, W.P. Quick, C. Labate, K. Fichtner, E.-D. Schulze, S.R. Rodermel, L. Bogorad and M. Stitt, Decreased ribulose-1,5-biphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' *rbc*S. VI. Effect on photosynthesis in plants grown at different irradiance, Planta, 190 (1993) 332–345.

[38] W.P. Quick, K. Fichtner, E.-D. Schulze, R. Wendler, R.C. Leegood, H. Mooney, S.R. Rodermel, L. Bogorad and M. Stitt, Decreased ribulose-1,5-biphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S. IV. Impact on photosynthesis in conditions of altered nitrogen supply, Planta, 188 (1992) 522–531.

[39] M. Stitt, W.P. Quick, U. Schurr, E.-D. Schulze, S.R. Rodermel and L. Bogorad, Decreased ribulose-1,5-biphosphate carboxylase-oxygenase in transgenic tobac-

co transformed with 'antisense' *rbc*S. II. Flux-control coefficients for photosynthesis in varying light, CO_2 and air humidity. Planta, 183 (1991) 555–566.

[40] W.P. Quick, U. Schurr, R. Scheibe, E.-D. Schulze, S.R. Rodermel, L. Bogorad and M. Stitt, Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S. I. Impact on photosynthesis in ambient growth conditions. Planta, 183 (1991) 542–554.

[41] K. Fichtner, W.P. Quick, E.-D. Schulze, H.A. Mooney, S.R. Rodermel, L. Bogorad and M. Stitt, Decreased ribulose-1,5-biphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S. V. Relationship between photosynthetic rate, storage strategy, biomass allocation and vegetative plant growth at three different nitrogen supplies. Planta, 190 (1993) 1–9.

[42] J. Masle, G.S. Hudson and M.R. Badger, Effects of ambient CO_2 concentration on growth and nitrogen use in tobacco (*Nicotiana tabacum*) plants transformed with an antisense gene to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol., 103 (1993) 1075–1088.

[43] G.D. Price, S. von Caemmerer, J.R. Evans, J.-W. Yu, J. Lloyd, V. Oja, P. Kell, K. Harrison, A. Gallagher and M.R. Badger, Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO_2 assimilation. Planta, 193 (1994) 331–340.

[44] D. Gunasekera and G.A. Berkowitz, Use of transgenic plants with ribulose-1,5-biphosphate carboxylase/oxygenase antisense DNA to evaluate the rate limitation of photosynthesis under water stress. Plant Physiol., 103 (1993) 629–635.

[45] C.-Z. Jiang, W.P. Quick, R. Allred, D. Kliebenstein and S.R. Rodermel, Antisense RNA inhibition of Rubisco activase expression. Plant J., 5 (1994) 787–798.

[46] C.J. Mate, G.S. Hudson, S. von Caemmerer, J.R. Evans and T.J. Andrews, Reduction of ribulose bisphosphate carboxylase activase levels in tobacco (*Nicotiana tobacum*) by antisense RNA reduces ribulose bisphosphate carboxylase carbamylation and impairs photosynthesis. Plant Physiol., 102 (1993) 1119–1128.

[47] D. Heineke, A. Kruse, U.-I. Flügge, W.B. Frommer, J.W. Riesmeier, L. Willmitzer and H.W. Heldt, Effect of antisense repression of the chloroplast triose-phosphate translocator on photosynthetic metabolism in transgenic potato plants. Planta, 193 (1994) 174–180.

[48] J.W. Riesmeier, U-I. Flügge, B. Schulz, D. Heineke, H.W. Heldt, L. Willmitzer and W.B. Frommer, Antisense repression of the chloroplast triose phosphate translocator affects carbon partitioning in transgenic potato plants. Proc. Natl. Acad. Sci. USA, 90 (1993) 6160–6164.

[49] J.W. Riesmeier, L. Willmitzer and W.B. Frommer, Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. EMBO J., 13 (1994) 1–7.

[50] M. Hajirezaei, U. Sonnewald, R. Viola, S. Carlisle, D. Dennis and M. Stitt, Transgenic potato plants with strongly decreased expression of pyrophosphate: fructose-6-phosphate phosphotransferase show no visible phenotype and only minor changes in metabolic fluxes in their tubers. Planta, 192 (1994) 16–30.

[51] N-G. Lee, B. Stein, H. Suzuki and D.P.S. Verma, Expression of antisense nodulin-35 RNA in *Vigna aconitifolia* transgenic root nodules retards peroxisome development and affects nitrogen availability to the plant. Plant J., 3 (1993) 599–606.

[52] J.N.M. Mol, A.R. Stuitje and A. van der Krol, Genetic manipulation of floral pigmentation genes. Plant Mol. Biol., 13 (1989) 287–294.

[53] A.R. van der Krol, L.A. Mur, P. de Lange, A.G.M. Gerats, J.N.M. Mol and A.R. Stuitje, Antisense chalcone synthase genes in petunia: Visualization of variable transgene expression. Mol. Gen. Genet., 220 (1990) 204–212.

[54] B. Müller-Röber, U. Sonnewald and L. Willmitzer, Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J., 11 (1992) 1229–1238.

[55] R. Zrenner, L. Willmitzer and U. Sonnewald, Analysis of the expression of potato uridinediphosphate-glucose pyrophosphorylase and its inhibition by antisense RNA. Planta, 190 (1993) 247–252.

[56] C.R. Bird, J.A. Ray, J.D. Fletcher, J.M. Boniwell, A.S. Bird, C. Teulieres, I. Blain, P.M. Bramley and W. Schuch, Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. BioTechnology, 9 (1991) 635–639.

[57] P. Bramley, C. Teulieres, I. Blain, C. Bird and W. Schuch, Biochemical characterization of transgenic tomato plants in which carotenoid synthesis has been inhibited through the expression of antisense RNA to pTOM5. Plant J., 2 (1992) 343–349.

[58] R.E. Sheehy, M. Kramer and W.R. Hiatt, Reduction of polygalacturonase activity in tomato fruit by antisense RNA. Proc. Natl. Acad. Sci. USA, 85 (1988) 8805–8809.

[59] M. Bouzayen, A. Hamilton, S. Picton, S. Barton and D. Grierson, Identification of genes for the ethylene-forming enzyme and inhibition of ethylene synthesis in transgenic plants using antisense genes. Biochem. Soc. Trans., 20 (1992) 76–79.

[60] A.J. Hamilton, G.W. Lycett and D. Grierson, Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature, 346 (1990) 284–287.

[61] T.A. Holton, F. Brugliera and Y. Tanaka, Cloning and expression of flavonol synthase from *Petunia hybrida*. Plant J., 4 (1993) 1003–1010.

[62] B.A. Sherf, A.M. Bajar and P.E. Kolattukudy, Abolition of an inducible highly anionic peroxidase activity in transgenic tomato. Plant Physiol., 101 (1993) 201–208.

[63] J.P. Gogarten, J. Fichmann, Y. Braun, L. Morgan, P. Styles, S.L. Taiz, K. DeLapp and L. Taiz, The use of antisense mRNA to inhibit the tonoplast H^+ ATPase in carrot. Plant Cell, 4 (1992) 851–864

[64] T. Fujiwara, P.A. Lessard and R.N. Beachy, Seed-specific repression of GUS activity in tobacco plants by antisense RNA. Plant Mol. Biol., 20 (1992) 1059–1069.

[65] U. Commandeur, W. Jarausch, Y. Li, R. Koenig and W. Burgermeister, cDNAs of beet necrotic yellow vein virus RNAs 3 and 4 are rendered biologically active in a plasmid containing the cauliflower mosaic 35S promoter. Virology, 185 (1991) 493–495.

[66] Z.R. Liu and J.C. Sanford, Investigation of the mechanism underlying the inhibitory effect of heterologous ras genes in plant cells. Plant Mol. Biol., 22 (1993) 751–765.

[67] D. Grierson and W. Schuch, Control of ripening. Philos. Trans. Royal Soc. Lond. B., 342 (1993) 241–250.

[68] W. Schuch, C.R. Bird, J. Ray, C.J.S. Smith, C.F. Watson, P.C. Morris, J.E. Gray, C. Arnold, G.B. Seymour, G.A. Tucker and D. Grierson, Control and manipulation of gene expression during tomato fruit ripening. Plant Mol. Biol., 13 (1989) 303–311.

[69] M. Kramer, R.E. Sheehy and W.R. Hiatt, Progress towards the genetic engineering of tomato fruit softening. Trends Biol. Technology, 7 (1989) 191–194.

[70] P.W. Oeller, L. Min-Wong, L.P. Taylor, D.A. Pike and A. Theologis, Reversible inhibition of tomato fruit senescence by antisense RNA. Science, 254 (1991) 437–439.

[71] R.G. Fray and D. Grierson, Molecular genetics of tomato fruit ripening. Trends Genet., 9 (1993) 438–443.

[72] C.J.S. Smith, C.F. Watson, P.C. Morris, C.R. Bird, G.B. Seymour, J.E. Gray, C. Arnold, G.A. Tucker, W. Schuch, S. Harding and D. Grierson, Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Mol. Biol., 14 (1990) 369–379.

[73] C.M.S. Carrington, L.C. Greve and J.M. Labavitch, Cell wall metabolism in ripening fruit. VI. Effect of the antisense polygalacturonase gene on cell wall changes accompanying ripening in transgenic tomatoes. Plant Physiol., 103 (1993) 429–434.

[74] D.M. Tieman, R.W. Harriman, G. Ramamohan and A.K. Handa, An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. Plant Cell, 4 (1992) 667–679.

[75] L. Peñarrubia, M. Aguilar, L. Margossian and R.L. Fischer, An antisense gene stimulates ethylene hormone production during tomato fruit ripening. Plant Cell, 4 (1992) 681–687.

[76] L.N. Hall, G.A. Tucker, C.J.S. Smith, C.F. Watson, G.B. Seymour, Y. Bundick, J.M. Boniwell, J.D. Fletcher, J.A. Ray, W. Schuch, C.R. Bird and D. Grierson, Antisense inhibition of pectin esterase gene expression in transgenic tomatoes. Plant J., 3 (1993) 121–129

[77] S. Picton, S.L. Barton, M. Bouzayen, A.J. Hamilton and D. Grierson, Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J., 3 (1993) 469–481.

[78] J. Gray, S. Picton, J. Shabbeer, W. Schuch and D. Grierson, Molecular biology of fruit ripening and its manipulations with antisense genes. Plant Mol. Biol., 19 (1992) 69–87.

[79] P. Elomaa, J. Honkanen, R. Puska, P. Seppänen, Y. Helariutta, M. Mehto, M. Kotilainen, L. Nevalainen and T.H. Teeri, *Agrobacterium*-mediated transfer of antisense chalcone synthase cDNA to *Gerbera hybrida* inhibits flower pigmentation. Bio/Technology, 11 (1993) 508–511.

[80] N. Courtney-Gutterson, C. Napoli, C. Lemieux, A. Morgan, E. Firoozabady and K.E.P. Robinson, Modification of flower color in florist's chrysanthemum: Production of a white-flowering variety through molecular genetics. Bio/Technology, 12 (1994) 268–271.

[81] B. McGurl, G. Pearce, M. Orozco-Cardenas and C.A. Ryan, Structure, expression, and antisense inhibition of the systemin precursor gene. Science, 255 (1992) 1570–1573.

[82] M. Orozco-Cardenas, B. McGurl and C.A. Ryan, Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. Proc. Natl. Acad. Sci. USA, 90 (1993) 8273–8276.

[83] J.-M. Neuhaus, S. Flores, D. Keefe, P.Ahl-Goy and F. Meins, Jr., The function of vacuolar β-1,3,-glucanase investigated by antisense transformation. Susceptibility of transgenic *Nicotiana sylvestris* plants to *Cercospora nicotianae* infection. Plant Mol. Biol., 19 (1992) 803–813.

[84] R.S. Beffa, J.-M. Neuhaus and F. Meins, Jr., Physiological compensation in antisense transformants: Specific induction of an "ersatz" glucan endo-1,3-β-glucosidase in plants infected with necrotizing viruses. Proc. Natl Acad Sci. USA, 90 (1993) 8792–8796.

[85] D.S. Knutzon, G.A. Thompson, S.E. Radke, W.B. Johnson, V.C. Knauf and J.C. Kridl, Modification of *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. Proc. Natl. Acad. Sci. USA, 89 (1992) 2624–2628.

[86] H-S. Lee, S. Huang and T-h. Kao, S proteins control rejection of incompatible pollen in *Petunia inflata*. Nature, 367 (1994) 560–563.

[87] T. Schmülling, H. Röhrig, S. Pilz, R. Walden and J. Schell, Restoration of fertility by antisense RNA in genetically engineered male sterile tobacco plants. Mol. Gen. Genet., 237 (1993) 385–394.

[88] H. Zhang, D.C. Scheirer, W.H. Fowle and H.M. Goodman, Expression of antisense or sense RNA of an ankyrin repeat-containing gene blocks chloroplast differentiation in *Arabidopsis*. Plant Cell, 4 (1992) 1575–1588.

[89] K.J.M. Boot, B.J. van der Zaal, J. Velterop, A. Quint, A.M. Mennes, P.J.J. Hooykaas and K.R. Libbenga, Further characterization of expression of auxin-induced genes in tobacco (*Nicotiana tabacum*) cell-suspension cultures. Plant Physiol., 102 (1993) 513–520.

[90] M. Cannon, J. Platz, M. O'Leary, C. Sookdeo and F. Cannon, Organ-specific modulation of gene expression in transgenic plants using antisense RNA. Plant Mol. Biol., 15 (1990) 39–47.

[91] C.G. Simpson and J.W.S. Brown, Efficient splicing of an AU-rich antisense intron sequence. Plant Mol. Biol., 21 (1993) 205–211.

[92] J.W.S. Brown, C.G. Simpson, G.G. Simpson, A.D. Tumbull-Ross and G.P. Clark, Plant pre-mRNA splic-

ing and splicing components. Phil. Trans. Royal Soc. Lond. B., 342 (1993) 217–224.
[93] P.D. Nagy and J.J. Bujarski, Targeting the site of RNA-RNA recombination in brome mosaic virus with antisense sequences. Proc. Natl. Acad. Sci. USA, 90 (1993) 6390–6394.
[94] R. Palomares, R.G. Herrmann and R. Oelmüller, Antisense RNA for components associated with the oxygen-evolving complex and the Rieske iron/sulfur protein of the tobacco thylakoid membrane suppresses accumulation of mRNA, but not of protein. Planta, 190 (1993) 305–312.
[95] J. Stockhaus, M. Höfer, G. Renger, P. Westhoff, T. Wydrzynski and L. Willmitzer, Anti-sense RNA efficiently inhibits formation of the 10 kDa polypeptide of photosystem II in transgenic plants: analysis of the role of the 10 kDa protein. EMBO J., 9 (1990) 3013–3021.
[96] S.R. Rodermel, M.S. Abbott and L. Bogorad, Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose biphosphate carboxylase enzyme levels in transformed tobacco plants. Cell, 55 (1988) 673–681.
[97] R. Höfgen, K.B. Axelsen, C.G. Kannangara, I. Schüttke, H-D. Pohlenz, L. Willmitzer, B. Grimm and D. von Wettstein, A visible marker for antisense mRNA expression in plants: Inhibition of chlorophyll synthesis with a glutamate-1-semialdehyde aminotransferase antisense gene. Proc. Natl. Acad. Sci. USA, 91 (1994) 1726–1730.
[98] C. Xiang and D.J. Guerra, The anti-*nptII* gene. Plant Physiol., 102 (1993) 287–293.
[99] M. Tabler and M. Tsagris, Catalytic antisense RNAs produced by incorporating ribozyme cassettes into cDNA. Gene, 108 (1991) 175–183.
[100] M.A. Rezaian, K.G.M. Skene and J.G. Ellis, Anti-sense RNAs of cucumber mosaic virus in transgenic plants assessed for control of the virus. Plant Mol. Biol., 11 (1988) 463–471.
[101] P.P. Abel, R.S. Nelson, B. De, N. Hoffman, S.G. Rogers, R.T. Fraley and R.N. Beachy, Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science, 232 (1986) 738–743.
[102] M. Cuozzo, K.M. O'Connell, W. Kaniewski, R-X. Fang, N-H. Chua and N.E. Tumer, Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. Bio/Technology, 6 (1988) 549–557.
[103] P.A. Powell, D.M. Stark, P.R. Sanders and R.N. Beachy, Protection against tobacco mosaic virus in transgenic plants that express tobacco mosaic virus antisense RNA. Proc. Natl. Acad. Sci. USA, 86 (1989) 6949–6952.
[104] G. Fang and R. Grumet, Genetic engineering of potyvirus resistance using constructs derived from the zucchini yellow mosaic virus coat protein gene. Mol. Plant-Microbe Interact., 6 (1993) 358–367.
[105] L. Farinelli and P. Malnoë, Coat protein gene-mediated resistance to potato virus Y in tobacco: examination of the resistance mechanisms — is the transgenic coat protein required for protection? Mol. Plant-Microbe Interact., 6 (1993) 284–292.
[106] C. Hemenway, R.-X. Fang, W.K. Kaniewski, N.-H. Chua and N.E. Tumer, Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO J., 7 (1988) 1273–1280.
[107] D.C. Baulcombe, W.D.O. Hamilton, M.A. Mayo and B.D. Harrison, in CIBA Foundation Symposium 133: Plant Resistances to Viruses, John Wiley, 1987, pp. 170–184.
[108] W.L. Gerlach, D. Llewellyn and J. Haseloff, Construction of plant disease resistance gene from the satellite RNA of tobacco ringspot virus. Nature, 328 (1987) 802–805.
[109] L.M. Kawchuk, R.R. Martin, J. McPherson, Sense and antisense RNA-mediated resistance to potato leafroll virus in russet burbank potato plants. Mol. Plant Microbe Interact., 4 (1991) 247–253.
[110] A. Nelson, D.A. Roth and J.D. Johnson, Tobacco mosaic virus infection of transgenic *Nicotiana tabacum* plants is inhibited by antisense constructs directed at the 5′ region of viral RNA. Gene, 127 (1993) 227–232.
[111] S-Z. Pang, J.L. Slightom and D. Gonsalves, Different mechanisms protect tobacco against tomato spotted wilt and impatiens necrotic spot *Tospoviruses*. Bio/Technology, 11 (1993) 819–824.
[112] J.A. Lindbo and W.G. Dougherty, Untranslatable transcripts of the tobacco, etch coat protein gene sequence can interfere with tobacco, etch virus replication in transgenic plants and protoplasts. Virology, 189 (1992) 725–733.
[113] A.G. Day, E.R. Bejarano, K.W. Buck, M. Burrell and C.P. Lichtenstein, Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. Proc. Natl. Acad. Sci. USA, 88 (1991) 6721–6725.
[114] E.R. Bejarano and C.P. Lichtenstein, Expression of TGMV antisense RNA in transgenic tobacco inhibits replication of BCTV but not ACMV geminiviruses. Plant Mol. Biol., 24 (1994) 241–248.
[115] A. von Arnim and J. Stanley, Inhibition of African cassava mosaic virus systemic infection by a movement protein from the related geminivirus tomato golden mosaic virus. Virology, 187 (1992) 555–564.
[116] C.C. Huntley and T.C. Hall, Interference with brome mosaic virus replication by targeting the minus strand promoter. J. Gen. Virol., 74 (1993) 2445–2452.
[117] A.J. Delauney, Z. Tabaeizadeh and D.P.S. Verma, A stable bifunctional antisense transcript inhibiting gene expression in transgenic plants. Proc. Natl. Acad. Sci USA, 85 (1988) 4300–4304.
[118] S.J. Sandler, M. Stayton, J.A. Townsend, M.L. Ralston, J.R. Bedbrook and P. Dunsmuir, Inhibition of gene expression in transformed plants by antisense RNA. Plant Mol. Biol., 11 (1988) 301–310.
[119] M. Cornelissen and M. Vandewiele, Both RNA level and translation efficiency are reduced by anti-sense RNA in transgenic tobacco. Nucl. Acids Res., 17 (1989) 833–843.
[120] P. de Lange, G-J. de Boer, J.N.M. Mol and J.M. Kooter,

Conditional inhibition of β-glucuronidase expression by antisense gene fragments in petunia protoplasts. Plant Mol. Biol., 23 (1993) 45–55.

[121] L.S. Robert, P.A. Donaldson, C. Ladaique, I. Altosaar, P.G. Arnison and S.F. Fabijanski, Antisense RNA inhibition of β-glucuronidase gene expression in transgenic tobacco plants. Plant Mol. Biol., 13 (1989) 399–409.

[122] A.R. van der Krol, L.A. Mur, P. de Lange, J.N.M. Mol and A. Stuitje, Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effects. Plant Mol. Biol., 14 (1990) 457–466.

[123] S.N.I.M. Salehuzzaman, E. Jacobsen and R.G.F. Visser, Isolation and characterization of a cDNA encoding granule-bound starch synthase in cassava (*Manihot esculenta* Crantz) and its antisense expression in potato. Plant Mol. Biol., 23 (1993) 947–962.

[124] A.R. van der Krol, J.N.M. Mol and A.R. Stuitje, Modulation of eukaryotic gene expression by complementary RNA or DNA sequences. Bio/Technology, 6 (1988) 958–975.

[125] A.R. van der Krol, J.N.M. Mol and A.R. Stuitje, Antisense genes in plants: an overview. Gene, 72 (1988) 45–50.

[126] L.S. Robert, P.A. Donaldson, C. Ladaique, I. Altosaar, P.G. Arnison and S.F. Fabijanski, Antisense RNA inhibition of β-glucuronidase gene expression in transgenic tobacco can be transiently overcome using a heat-inducible β-glucuronidase gene construct. Bio/Technology, 8 (1990) 459–464.

[127] W.M. Ainley and J.L. Key, Development of a heat shock inducible expression cassette for plants: Characterization of parameters for its use in transient assays. Plant Mol. Biol., 14 (1990) 949–967.

[128] I.M. van der Meer, M.E. Stam, A.J. van Tunen, J.N.M. Mol and A.R. Stuitje, Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell, 4 (1992) 253–262.

[129] J.E. Bourque and W.R. Folk, Suppression of gene expression in plant cells utilizing antisense sequences transcribed by RNA polymerase III. Plant Mol. Biol., 19 (1992) 641–647.

[130] P.A. Jennings and P.L. Molloy, Inhibition of SV40 replicon function by engineered antisense RNA transcribed by RNA polymerase III. EMBO J., 6 (1987) 3043–3047.

[131] Y. Eguchi, T. Itoh and J-i. Tomizawa, Antisense RNA. Ann. Rev. Biochem., 60 (1991) 631–652.

[132] H. Shimada, Y. Tada, T. Kawasaki and T. Fujimura, Antisense regulation of the rice waxy gene expression using a PCR-amplified fragment of the rice genome reduces the amylose content in grain starch. Theor. Appl. Genet., 86 (1993) 665–672.

[133] S.J. Temple, T.J. Knight, P.J. Unkefer and C. Sengupta-Gopalan, Modulation of glutamine synthetase gene expression in tobacco by the introduction of an alfalfa glutamine synthetase gene in sense and antisense orientation: molecular and biochemical analysis. Mol. Gen. Genet., 236 (1993) 315–325.

[134] R.G.F. Visser, I. Semhorst, G.J. Kuipers, N.J. Ruys, W.J. Feenstra and E. Jacobsen, Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. Mol. Gen. Genet., 225 (1991) 289–296.

[135] R.G.F. Visser, W.J. Feenstra and E. Jacobsen, Manipulation of granule-bound starch synthase activity and amylose content in potato by antisense genes, in: J.N.M. Mol and A.R. van der Krol (Eds.), Antisense Nucleic Acids and Proteins: Fundamentals and Applications, Marcel Dekker, New York, 1990, pp. 141–155.

[136] L.J. Chang and C.M. Stoltzfus, Gene expression from both intronless and intron-containing Rous sarcoma virus clones is specifically inhibited by anti-sense RNA. Mol. Cell Biol., 5 (1985) 2341–2348.

[137] D.A. Melton, Injected anti-sense RNAs specifically block messenger RNA translation in vivo. Proc. Natl. Acad. Sci. USA, 82 (1985) 144–148.

[138] C.C. Smith, L. Aurelian, M. Reddy, P.S. Miller and P.O.P. Ts'O, Antiviral effects of an oligo(nucleoside methylphosphate) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5. Proc. Natl. Acad. Sci. USA, 83 (1986) 2787–2791.

[139] J. Mol, R. van Blokland and J. Kooter, More about co-suppression. Trends Biotechnol., 9 (1991) 182–183.

[140] A.R. van der Krol, A.R. Stuitje and J.N.M. Mol, Modulation of floral pigmentation by antisense technology, in: J.N.M. Mol and A.R. van der Krol (Eds.), Antisense Nucleic Acids and Proteins: Fundamentals and Applications, Marcel Dekker, New York, 1991, pp. 125–140.

[141] A. Hamilton and D. Grierson, ACC-oxidase: Investigation of mechanism. Proceedings of the 4th International Congress of Plant Mol. Biol., 3–1 (1994) 492. The Netherlands.

[142] C. Crum, J.D. Johnson, A. Nelson and D. Roth, Complementary oligodeoxynucleotide mediated inhibition of tobacco mosaic virus RNA translation in vitro. Nucl. Acids Res., 16 (1988) 4569–4581.

[143] B.L. Bass and H. Weintraub, A developmentally regulated activity that unwinds double-stranded RNA duplexes. Cell, 48 (1987) 607–613.

2002 WL 31234529 (Bd.Pat.App & Interf.)
(Cite as: 2002 WL 31234529 (Bd.Pat.App & Interf.))

*1 THIS OPINION WAS NOT WRITTEN FOR PUBLICATION

Board of Patent Appeals and Interferences

Patent and Trademark Office (P.T.O.)
EX PARTE GERMAIN FUH AND JAMES A. WELLS
Appeal No. 1999-1732
Application No. 08/308,879

NO DATE REFERENCE AVAILABLE FOR THIS DOCUMENT

GATES and COOPER

HOWARD HUGHES CENTER

670 CENTER DRIVE WEST

SUITE 1050

LOS ANGELES, CA 90045

Before WINTERS, SCHEINER and GREEN

Administrative Patent Judges

GREEN

Administrative Patent Judge

ON BRIEF

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 1 and 3-7. Claim 1 is representative of the subject matter on appeal, and reads as follows:

1. A method for inhibiting the growth of breast cancer cells expressing prolactin receptors comprising contacting the cells with an effective amount of a growth hormone analog, wherein the analog is an antagonist which binds to the prolactin receptor and the analog is a variant of a naturally occurring growth hormone which includes an amino acid variation that reduces growth hormone receptor binding at site two by at least two-fold in relation to native growth hormone.

The examiner relies on the following references:

Kopchick et al. (Kopchick) 5,350,836 Sep. 27, 1994

Phares, "Regression of Rat Mammary Tumors Associated with Suppressed Growth

Hormone," Anticancer Research, Vol. 6, pp. 845-848 (1986).

Watahiki et al. (Watahiki), "Conserved and Unique Amino Acid Residues in the Domains of the Growth Hormones," J. Biol. Chem., Vol. 264, pp. 312-316 (1989).

Chen et al. (Chen), "Expression of a mutated bovine growth hormone gene suppresses growth of transgenic mice," Proc. Natl. Acad. Sci., Vol. 87, pp. 5061-5065 (1990).

Claims 1 and 3-7, which stand or fall together, stand rejected under 35 U.S.C. § 103(a) as being obvious over Kopchick. In addition, claims 1 and 3-7 stand rejected under 35 U.S.C. § 103(a) over the combination of Chen, Phares and Watahiki. After careful review of the record before us, we reverse both rejections.

BACKGROUND

Growth hormone is a member of a homologous hormone family that also includes prolactins, placental lactogens, and other genetic and species variants of growth hormone. Human growth hormone not only binds to its own receptor, but can also bind to either cloned somatogenic or prolactin receptors. See Specification, page 3. The human growth hormone sequence is known, and the hormone has been cloned. See id. at pages 3-4.

Prolactin and growth hormone are known to play a role in the development and progression of breast cancer. The majority of breast cancer cells overexpress the prolactin receptor, and human breast cancer cell lines have been shown to respond to both prolactin and growth hormone when grown as solid tumors in nude mice. See id. at page 5.

***2** Growth hormone and the class of conformational ligands to which they belong form a 1:2 complex with their receptor, with a first ligand binding site, which the specification refers to as site 1, binds to a first receptor, and then a second receptor binds to the hormone at the second ligand site, site 2. See id. at page 6. Coupled with the knowledge of the conformational structure of the ligand, the specification states that one can design hormone agonists or antagonists by introducing amino acid variations into sites 1 and/or 2. See id. at pages 6-7. The specification states that

> [i]n particular, antagonists for polypeptide ligands are provided which comprise an amino acid sequence mutation in site 2 which reduces or eliminates the affinity of the ligand for the receptor at site 2. Ideally, the ligand antagonist analog will have low or no affinity for receptor at site 2 and will have elevated affinity for receptor at site 1.

Id. at page 7.

The claimed invention is drawn to a method for inhibiting growth of breast cancer cells through the use of a growth factor antagonist, wherein the antagonist has an amino acid variation that reduces ligand binding at site 2 by at least two-fold. Also claimed is a preferred antagonist, wherein the glycine at position 120 of human growth hormone has been mutated to arginine (G120R).

DISCUSSION

The answer contains two grounds of rejection, both based on obviousness. We will

address the obviousness rejection in reverse order, addressing the rejection over Kopchick first.

The Answer relies on the Kopchick reference for teaching growth hormone receptor antagonists, and specifically for teaching that the mutant in which the glycine at position 120 has been mutated to arginine (G120R) is a growth receptor antagonist. With respect to the use of using such growth hormone antagonists to treat breast cancer, the Kopchick reference states:

It has been suggested that long-activity somatostatin analogues may have value in the control of breast and prostate cancers. Manni, Biotherapy, 4:31- 36 (1992). Manni hypothesizes that they could inhibit tumor growth by a number of mechanisms, including inhibiting growth hormone secretion. Growth hormone is implicated because it is lactogenic and because it elevates IGF-1 levels. We suggest that the growth hormone antagonists of the present invention may be used in the treatment of cancers whose growth is facilitated by endogenous growth hormone or IGF-1. Kopchick, Col. 3, line 60-Col. 4, line 2 (emphasis added). The reference, however, presents no examples, nor does it provide in vitro or in vivo data, wherein the disclosed growth hormone antagonists are administered to breast cancer cells that express prolactin receptors, as required by claim 1.

Based on this single paragraph in the Kopchick reference, the Answer concludes that "it would have been obvious to a person of ordinary skill in the art to administer [growth hormone] antagonists such as [G120R] to mammary tumor cells or patients having breast cancer to regress tumor growth because Kopchick et al. teach that [growth hormone] antagonists are useful in the treatment of breast cancer. See Answer, pages 5-6.

*3 Appellants argue that the statements in the Kopchick reference are merely an invitation to experiment, or that it is merely obvious to try the process suggested by the reference, and thus that the reference does not provide a reasonable expectation of success of inhibiting the growth of breast cancer cells using a growth hormone antagonist. We agree.

A determination of obviousness not only requires that the prior art would have suggested the claimed process to one of ordinary skill in the art, but also that the process would have a reasonable likelihood of success when viewed in light of the prior art. See In re Dow Chemical Co., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). A rejection based on a reference or a combination of references amounts to an "invitation to experiment," and is thus "obvious-to-try," "when a general disclosure may pique the scientist's curiosity, such that further investigation might be done as a result of the disclosure, but the disclosure itself does not contain a sufficient teaching of how to obtain the desired result, or that the claimed result would be obtained if certain directions were pursued." In re Eli Lilly & Co., 902 F.2d 943, 945, 14 USPQ2d 1741, 1743 (Fed. Cir. 1990). The Kopchick reference, while suggesting that growth hormone antagonists may be used in the treatment of cancers whose growth is facilitated by endogenous growth hormone, does not contain a sufficient teaching that the claimed result would be obtained. Thus, the answer fails to set forth a prima facie case that the claims are rendered obvious by the Kopchick reference.

The treatment of cancers, such as breast cancer, is an unpredictable art. The somatostatin analogues discussed by Kopchick are hypothesized to inhibit tumor growth by inhibiting growth hormone secretion. See Kopchick, col. 3, lines 63- 65.

But that is only one of a number of hypothesized mechanisms by which somatostatin analogues may inhibit tumor growth. Even if somatostatin analogues act to inhibit tumor growth by suppressing serum growth hormone levels, growth hormone antagonists, however, actually increase the production of endogenous growth hormone in the pituitary, and may lead to increased serum growth hormone levels. See Cronin Declaration, page 6. Therefore, the growth hormone antagonists and the somatostatin analogues work by different mechanisms, and thus the ordinary artisan would not have a reasonable expectation of success from data produced by somatostatin analogues in inhibiting breast cancer growth to translate to success with growth hormone antagonists.

The claims were also rejected as obvious over the teachings of the combined teachings of Phares, Chen and Watahiki.

The Phares reference uses a growth hormone agonist, pleroceroid growth factor (PGF) to study the effect of endogenous growth hormone levels on the growth of 7, 12, dimethylbenz(a)anthracene (DMBA)- or N-nitrosomethylurea (NMU)-induced rat mammary tumors in vivo. Phares found that PGF caused regression of most of the mammary tumors induced by DMBA or NMU, and that endogenous growth hormone serum levels were also reduced. The Answer also relied on Phares for teaching that growth hormone has been implicated as a growth stimulant for rat mammary tumors and for human breast cancer cells, which may be due to its regulatory influence prolactin receptors. The reference also states that NMU-induced mammary tumors are regressed by the inhibition of growth hormone release from the hypothesis with somatostatin, leading to the conclusion that growth hormone plays an active role in mammary tumor formation. The Answer acknowledges that the reference does not teach the use of a growth hormone receptor antagonist to regress mammary tumor cell growth, but asserts that a "routineer would reasonably expect that the removal of [growth hormone] influence at the [prolactin] and estrogen receptors located on mammary tumor cells will cause tumor regression because decreased serum levels of [growth hormone] and, then, reduced activity at the receptors, has been shown to regress mammary tumors." Answer, pages 4-5.

*4 The Answer characterizes Chen as teaching a bovine growth hormone antagonist, wherein three substitutions have been made. Watahiki is then relied upon for teaching the identity of growth hormones across several species, and that if the mutations had been made to human growth hormone, one of the substitutions would have been G120R.

The rejection concludes that it would have been obvious to the ordinary artisan to substitute the PGF as used by Phares to reduce growth hormone levels with the growth hormone antagonist taught by Chen "because the antagonist would be expected to reduce [growth hormone] activity or influence at the mammary tumor cell [prolactin] and estrogen receptors like the reduced serum [growth hormone] levels found after PGF." Answer, page 5. Because Phares teaches that NMU-induced rat mammary tumors are regressed with decreased growth hormone influence, the Answer also concludes that there is a reasonable expectation of success that receptor antagonism would be useful for the treatment of breast cancer.

Appellants put forth several arguments why the combination of Phares, Chen and Watahiki do not render the claimed process of inhibiting the growth of breast cancer cells obvious. In particular, Appellants argue that at most, the combination provides an invitation to experiment, and thus does not produce a reasonable

expectation of success. Again, we agree, for basically the same reasons discussed above with respect to the rejection over Kopchick.

As pointed out by Appellants, the PGF used by Phares to treat NMU-induced rat mammary tumors is a growth hormone agonist. As with the somatostatin analogues, growth hormone agonists decrease serum growth hormone levels. In contrast, as explained in the expert declaration, growth hormone antagonists may actually increase serum growth hormone levels. Moreover, the differences in mechanism between the growth hormone agonist PGF and growth hormone antagonists are also demonstrated by the fact that PGF treated animals demonstrate increased growth, whereas growth hormone antagonist treated animals have decreased levels of growth. Thus, there is no reasonable expectation that replacing the growth hormone agonist of Phares with the postulated growth hormone antagonist taught by Chen would result in inhibiting the growth of breast cancer cells.

The Answer asserts that one of ordinary skill in the art would have understood that Phares teaches that PGF reduces serum growth hormone, and that it would have been obvious to a routineer that a growth hormone antagonist would have the same effect--to block or prevent growth hormone from binding to the receptor. It is unpredictable, however, what effect that the antagonist will have at the receptor, as demonstrated by the fact that in Phares, the experimental animals treated with PGF experienced an increase in growth, whereas the instant specification teaches that animals treated with growth hormone antagonist have decreased growth. Thus, while Phares may have provided the ordinary artisan incentive to try the use of a growth hormone antagonist to reduce the growth of breast cancer cells, because of the different mechanisms and the different receptors that may be involved, the reference indeed does not provide a reasonable expectation of success of achieving the claimed result-- inhibiting the growth of breast cancer cells.

CONCLUSION

***5** For the reasons stated above, the rejections of claims 1 and 3-7 under 35 U.S.C. § 103(a) are reversed.

REVERSED

BOARD OF PATENT APPEALS AND INTERFERENCES

SHERMAN D. WINTERS

Administrative Patent Judge

TONI R. SCHEINER

Administrative Patent Judge

LORA M. GREEN

Administrative Patent Judge

LMG/jlb

END OF DOCUMENT

ROBERT LOUIS FINKEL
ATTORNEY AT LAW
PATENTS, TRADEMARKS, COPYRIGHTS & RELATED CAUSES
5215-2 White Oak Avenue, Encino, California 91316
P. O. Box 19276
Encino, California 91416-9276
(818) 705-9861
FAX (818) 705-7811
belchfire@sprintmail.com

DATE: 8-11-04

TO: Commissioner for Patents
Attention: Art Unit 2859

FROM: Robert Louis Finkel

SUBJECT: App. No. 10,856,922

ENCLOSED: Information Disclosure Statement and attachment; check

Enclosed for filing in connection with the above identified application is an Information Disclosure Statement by Applicant, citing nine U.S. patent documents and one foreign patent document, together with the undersigned's check No. 14525 in the amount of $180.00.

Respectfully submitted,

Robert Louis Finkel
Attorney for Applicant

RLF/k
Enclosures
IDS.TTL

ROBERT LOUIS FINKEL
ATTORNEY AT LAW
P.O. BOX 19276
ENCINO, CA 91416-9276
(818) 705-9861

WELLS FARGO BANK, N.A.
ENCINO, CA 91436
16-24/1220

14525

8-11-04

PAY TO THE ORDER OF Commissioner for Patents $ 180.00

One hundred eighty and 00/100 - - - - - - - - - - - - - - - - DOLLARS

MEMO IDS 10/856,922 Polly

⑈014525⑈ ⑆122000247⑆06170887580⑈

USPTO/~~COCt~~ Commissioner for Patents Date 8-11-04
SN/~~Pat No/Reg No/Case No~~ 10,856,922
Filing Date 7-19-04
Inventor/~~Applicant~~/Title/~~Mark/Caption~~ John R. Polly
BOOKMARK FOR OPENING A BOOK TO A SELECTED PAGE

Examiner Art Unit 2859 Grp/Div/Sec/Law Off
Application Chk No 14525 $180
Assignment No of Pgs
Response to Off Action No of Clms
Issue Fee Sheets of Drwgs
Not of Appeal Decl/Oath
Brief
Req Ext Time DATE RECEIVED
Other Information Disclosure Statement by Applicant
Reference

PTO/SB/08a (08-03)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 4

Complete if Known	
Application Number	10/856,922
Filing Date	07/19/2004
First Named Inventor	John R. Polly
Art Unit	2859
Examiner Name	
Attorney Docket Number	JRP-001US

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-3,143,998	08-11-1964	Madden	See attached sheet
		US-5,022,342	06-11-1991	Davis	
		US-5,375,884	12-27-1994	Farrington	
		US-5,439,254	08-08-1995	Dorion	
		US-5,515,809	05-14-1996	Weinberg	
		US-6,109,204	08-29-2000	Hoey	
		US-2002-0158462	08-31-2002	Antoine	
		US-D314,589	02-12-1991	Cooley	
		US-D453,185	01-29-2002	Kehrlein	
		US-			
		US-			
		US-			
		US-			
		US-			
		US-			
		US-			
		US-			
		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		PCT/US82/01104	02-17-83	Zeisky		

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

Application Number	**10/856,922**	)
Filling Date	**07/19/2004**	)
Inventor	**Polly, John R.**	)
Art Unit	**2859**	)
Examiner	**Not assigned**	)
Attorney Docket Number	**JRP-001US**	)

ATTACHMENT TO INFORMATION DISCLOSURE STATEMENT BY APPLICANT

The following synopses summarize the pertinent features disclosed by the references cited in the foregoing Information Disclosure Statement and indicate the pages, columns and lines where relevant passages or figures appear.

Document Number	Pertinence
3,143,998	Discloses and illustrates a bookmark 10 comprising a clip member 11 adapted to be attached to the back of a book cover or inserted in the binding. A page marker disc 18 is connected with the clip 11 by means of a flexible connector member 19 as, for example, a piece of string, cord or nylon line. As stated in Column 2, Lines 59-62, ". . .connector member 19 is provided with sufficient length to permit the reader to place the page marker 18 well down near the bottom of the book between the two pages. . ."
5,022,342	Discloses and illustrates an apparatus (see Fig. 3) having an anchor 104 comprising a Velcro®-type hook and loop fastener glued to the outside spine 150 of a book. A string 120 (or a plurality of strings) attached to one of the Velcro® members has a pair of moveable place markers 126 adapted to allow the reader to mark places of interest on the page, and knot 130 at the end of the string

to retain the markers. Other than the suggestion that the markers 126 may take the form of beads, no reference is made to the use of a spreader to facilitate opening the book to the desired pages.

5,375,884 Discloses and illustrates a one-piece bookmark made of a flexible material, like molded plastic, having an attachment portion employing a U-shaped clip or other unspecified attachment means for removably attaching the bookmark to a book, a long, narrow marking section 28 having an end portion that may be plain or may consist of a decorative shape such as a round medallion or useful object, and a bendable thread section linking the attachment and marking portions. No specific mention is made of the use of a material like Velcro® or the provision of an enlarged spreader or other means to facilitate opening the book at the desired place.

5,439,254 Discloses and illustrates a flexible paper bookmarker adapted to be attached adhesively to the spine of a book for insertion between the book's pages. The patent deals primarily with a method for making a bookmark out of a one-piece fiber reinforced paper which can be die cut in quantity and provided with a surface which can be printed or written upon. (See Abstract; Col. 2, lines 37-40.)

5,515,809 Discloses and illustrates various forms of a bookmark having a removable adhesive at one end for attachment to the spine of a

book and a variety of markers adhesively attached to an elongated flexible tail member, allowing the reader to selectively mark the page. (See Col. 5, lines 28, 32-34.)

6,109,204 Discloses and illustrates a number of alternative means for removably attaching marking means 24 such as a rigid wire or flexible ribbon, fabric, yarn or string and may be elastic or inelastic.

2002/0158462 Discloses and illustrates a more or less conventional bookmarker which can be removably secured to the spine of a book by means of a pair of tabs separated by a fold line, one of the tabs bearing an adhesive for attachment to the spine of a book. (See Abstract; Paragraph 0012.)

D314,589 Discloses an ornamental design for a bookmark having what appears to be an adhesive applied to its upper surface for attachment to the inside of the spine of a bound book.

D453,185 Discloses the ornamental design for a ribbon bookmark as shown.

PCT/US82/01104 Discloses and illustrates a clip-on bookmark having resilient means for fastening onto the spine or pages of a book. (See Abstract.)